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(71) Applicant (for all designated States except US): OXFORD BIOMEDICA (UK) LIMITED [GB/GB]; Medawar Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA (GB).			
(72) Inventors; and (75) Inventors/Applicants (for US only): LEWIS, Claire [GB/GB]; University of Sheffield, Dept. of Pathology, Beech Hill Road, Sheffield S10 2RX (GB). BINLEY, Katie, Mary [GB/GB]; 17 Demesne Furze, Headington, Oxford OX3 7XF (GB). BEBBINGTON, Chris [GB/GB]; Berry Cottage, Westbrook, Boxford, Newbury RG20 8DJ (GB). NAYLOR, Stuart [GB/GB]; 64 Woodside Road, Amersham, Bucks HP6 6AN (GB).			
(74) Agents: HARDING, Charles, Thomas et al.; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).			

(54) Title: EXPRESSION OF GENES IN HEMATOPOIETIC STEM CELLS IN HISCHAEMIC CONDITIONS

## (57) Abstract

A modified haematopoietic stem cell (MHSC) is described. The MHSC comprises at least one expressable nucleotide sequence of interest (NOI) wherein the or each NOI is operably linked to one or more ischaemia like response element (ILRE).

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## EXPRESSION OF GENES IN HEMATOPOIETIC STEM CELLS IN HISCHAEMIC CONDITIONS

The present invention relates to a method. In particular, the present invention relates to a method for the delivery of a nucleotide sequence of interest (NOI) to a haematopoietic stem cell (HSC).

This invention also relates to the use of vectors for the delivery of a nucleotide sequence of interest (NOI) to a haematopoietic stem cell (HSC).

Gene transfer involves the delivery to target cells, such as HSCs, of an expression cassette made up of one or more NOIs and the sequences controlling their expression. This can be carried out *ex vivo* in a procedure in which the cassette is transferred to cells in the laboratory and the modified cells are then administered to a recipient. Alternatively, gene transfer can be carried out *in vivo* in a procedure in which the expression cassette is transferred directly to cells within an individual. In both strategies, the transfer process is usually aided by a vector that helps deliver the cassette to the appropriate intracellular site.

The expression of a therapeutic gene at a targeted site can be regulated by promoter and enhancer elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (Maniatis *et al* 1987 Science 236: 1237). Promoter and enhancer elements have been isolated from a variety of eucaryotic sources including genes in yeast, insect, mammalian cells and viruses. The selection of a particular promoter and enhancer depends on what cell type is be used to express the protein/gene of interest.

A promoter may be functional in a variety of tissue types and in several different species of organisms or its function may be restricted to a particular species and/or a particular tissue. A promoter/enhancer may have a broad host range (such as SV40, human CMV) or it may be functional in a limited subset of cell types. Further, a promoter may be constitutively active or it may be selectively activated by certain substances (eg a tissue specific factor),

under certain conditions (eg hypoxia or the presence of an enhancer element) or during certain developmental stages of the organism (eg active in the fetus and silent in the adult). Bone marrow has been the traditional source of HSCs for transduction, more recent studies have suggested that peripheral blood stem cells or cord blood cells may be equally good or better target cells (Cassel *et al* 1993 Exp Hematol 21: 585-591; Bregni *et al* 1992 Blood 80: 1418-1422; Lu *et al* 1993 J Exp Med 178: 2089-2096).

Even more recently, potent stem cells have been extracted from an embryo cloned by nuclear transfer from fetal bovine fibroblasts (First and Thompson 1998 Nature Biotechnology 16: 620; Cohen 1998 New Scientist 11 July 4-5). When human embryonic stem cell lines become available, they will be capable of providing an unlimited source of *in-vitro* derived differentiated cells to treat specific diseases by gene therapy and/or transplantation.

Pretransduction enrichment for HSCs *via* positive selection for antigens such as CD34<sup>+</sup> or negative selection for lineage specific antigens has also been investigated. While it does not seem to greatly influence transduction efficiency it does allow much more practical volumes for *ex vivo* manipulation and transduction (Hughes *et al* 1992 *ibid*; Berenson *et al* 1988 J Clin Invest 81: 951-955).

It has been suggested that the low efficiency transfer to HSCs may be due to a lack of cell cycling and integration but could also be due to insufficient viral receptor density.

In attempts to surmount this problem, various strategies have been employed to target a viral vector to a particular site. These include: (i) modifying the envelope protein on the retroviral vector; (ii) using a promoter/enhancer to restricts expression to a particular site; and (iii) providing a vector with a ligand specific for a receptor on a target cell.

By way of example, a recombinant retroviral vector capable of targeting human cells expressing a c-Kit receptor *via* a ligand-receptor interaction has been engineered (Yajima *et al* 1998 Hum Gen Ther 10: 779-787). The ecotropic (Moloney murine leukemia virus)



envelope protein was modified by inserting a sequence encoding the N-terminal 161 amino acids of murine stem cell factor (mSCF). It has been suggested that this vector may prove useful for targeting cells expressing c-Kit on their surface.

5 By way of further example, other researchers (Fielding *et al* 1998 Blood 91: 1802-1809) have shown that *in vitro* cancer cells can be selectively transduced by a retroviral vector displaying stem cell factor (SCF) as part of a chimeric envelope glycoprotein whereas HSCs can be selectively transduced by a retroviral vector displaying epidermal growth factor (EGF) as part of its envelope.

10

In addition, HSCs have also been transduced with a viral vector bearing the vesicular stomatitis G (VSV-G). According to WO9609400, CD34<sup>+</sup> Thy-1<sup>+</sup> mobilised blood cells are transduced at surprisingly high efficiency by a VSV-G pseudotyped retroviral vector as compared with a CD34<sup>+</sup> adult bone marrow cells and as compared with the transduction  
15 efficiency of a conventional amphotropic vector.

The increasing ability to detect individuals within a population who are at an increased risk of developing cancer due to their genetic make-up (Cornelisse *et al* 1996 Pathol Res Pract. 192: 684-693) means that there is a need to provide prophylaxis in individuals who are  
20 particularly at risk from contracting these diseases. The same is true for individuals within a population who are genetically pre-disposed to coronary heart disease or rheumatoid arthritis or who have been exposed to plasmodium parasites which cause cerebral malaria, means that there is a need to provide prophylaxis in individuals who are particularly at risk from contracting these diseases.

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In particular, an increased understanding of the molecular genetics of cancer has led to the development of various novel therapeutic strategies for cancer. Gene-based therapies currently being tested in clinical trials involve largely the *ex vivo* or *in vivo* use of viral or liposomal vectors to deliver genes to tumours for: (i) tumour suppressor gene replacement  
30 or oncogene inactivation, (ii) the expression of cytokines/vaccines known to activate or enhance anti-tumour immune mechanisms, (iii) enhanced drug sensitivity (e.g. pro-drug

delivery or activation) (iv) drug resistance for bone marrow protection from high dose chemotherapy, and (v) inhibitors of tumour angiogenesis (Roth and Cristiano 1997 J Natl Cancer Inst 89: 21; Jaggar and Bicknell R 1997 In: 'Tumour Angiogenesis' Eds. Bicknell, Lewis and Ferrara pp357-372. Oxford University Press, Oxford. UK).

5

Targeting the expression of such therapeutic genes specifically to solid tumours has, until recently, been problematic. In some instances, recombinant viral vectors bearing therapeutic genes have been targeted to specific cell types by the insertion of ligands/antibodies in to the viral capsid. This approach requires the expression of tumour-  
10 antigens - as well as tissue-specific antigens by the malignant cell. Thus any therapy is restricted to use in particular patient groups and tumour types showing expression of the selected tumour antigen. Alternatively, naked or viral-incorporated DNA can be injected directly in to the tumour (or at least into the local blood supply for the tumour) to maximise specificity of delivery and expression at the tumour site. Although, this approach has met  
15 with limited success for superficial tumours (e.g. breast and melanoma lesions), it relies on the accurate localisation of the tumour and does not ensure DNA uptake by the entire tumour mass or treat secondary local or distant metastatic deposits (Roth and Cristiano 1997 J Natl Cancer Inst 89: 21).

20 Recently, an alternative approach for targeting therapeutic gene expression to tumours has been developed (Dachs *et al* 1997 Nature Med 5: 515). This utilises the abnormal physiology that exists in almost all solid tumours, regardless of their origin or location, and uses the tumour-specific condition of severe ischaemia, and its effects on specific enhancer regions of certain genes, to control the expression of heterologous genes (Dachs *et al* 1997  
25 *ibid*; UK Patent Application No. 9701975.6).

Aggressive tumours generally have insufficient blood supply, partly because tumour cells grow faster than the endothelial cells that make up the blood vessels, and partly because the newly formed vascular supply is disorganised (Vaupel 1993 In 'Drug Resistance in  
30 Oncology'. pp53-85. Ed. Teicher BA. Marel Dekker, New York). This results in areas of ischaemia and nutrient deprivation, including regions with both reduced oxygen tension

(hypoxia) and glucose. Oxygen electrode measurements of tumours have shown significant proportions of readings below 2.5 mmHg (normal tissues ranges from 24 to 66 mmHg) (Kallinowski 1996 The Cancer J. 9: 37). Moreover, hypoxic cells are markedly less sensitive to radiotherapy - and chemotherapy, which is why, in part, increased levels of tumour hypoxia correlate with reduced survival in many forms of cancer (Kallinowski 1996 *ibid*).

Thus, ischaemia is a general feature of solid tumours regardless of their cellular origin or patient population. It has recently been shown that it is possible to exploit tumour hypoxia to obtain selective expression of genes in tumours (Dachs 1997 *ibid*). Hypoxia is a powerful regulator of gene expression in a wide range of different cell types (Wang and Sememnza 1993 Proc Natl Acad Sci USA 90:4304) and acts by the induction of the activity of hypoxia-inducible transcription factors such as hypoxia inducible factor-1 (HIF-1) (Wang and Sememnza 1993 *ibid*), which bind to cognate DNA recognition sites, the hypoxia-response elements (HREs) on various gene promoters. Dachs *et al* (1997 *ibid*) used a multimeric form of the HRE from the mouse phosphoglycerate kinase-1 (PGK-1) gene (Firth *et al* 1994 Proc Natl Acad Sci USA 91: 6496) to control expression of both marker and therapeutic genes by human fibrosarcoma cells in response to hypoxia *in vitro* and within solid tumours *in vivo* (Dachs *et al* 1997 *ibid*). Alternatively, the fact that marked glucose deprivation is also present in ischaemic areas of tumours, HRE can be used to activate heterologous gene expression specifically in tumours. A truncated 632 base pair sequence of the *grp78* gene promoter, known to be activated specifically by glucose deprivation, has also been shown to be capable of driving high level expression of a reporter gene in murine tumours *in vivo* (Gazit *et al* 1995 Cancer Res. 55: 1660).

25

Ischaemic damage may also occur in many other tissues when the blood supply to the tissue is reduced or cut off. Stroke, deep vein thrombosis, pulmonary embolus and renal failure are examples of conditions that can cause such damage. The cell death of cardiac tissue, called myocardial infarction, is due in large part to tissue damage caused by ischemia and/or ischemia followed by reperfusion. Recurrent ischaemia and reperfusion typically results in oxidative damage to cells from reactive oxygen species. The extent and type of

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damage depends on the severity and nature of the hypoxic stress. For example, the stress may cause tissue necrosis. Alternatively, the stress may initiate apoptosis (programmed cell death) to eliminate the damaged cells.

- 5 The pre-clinical studies have, to date, only used tumour cells transfected with marker or therapeutic genes to show their specific expression in tumours. (See reviews by Dunbar and Eammons 1994 Stem Cells 12: 563-576; Crystal 1995 Science 270: 404-410; Lee and Klein 1995 Transfusion Medicine II 9: 91-113). Thus, there remains the question of how best to introduce these constructs to solid tumours *in vivo* for gene therapy protocols.
- 10 Methods have also been described which exploit cells in the immune system called macrophages as a delivery vehicle for targeting drugs and therapeutic genes to solid tumours (UK Patent Application No. 9701975.6; UK Patent Application No. 9620952.3). It has been shown that macrophages, derived from monocytes from the bloodstream, continually enter solid tumours and congregate in poorly vascularised, ischaemic sites in
- 15 breast carcinomas (Leek *et al* 1996 Cancer Res. 56: 4625). Moreover, the degree of ischaemia-induced necrosis in these tumours was positively correlated with the degree of intra-tumoral macrophage infiltration (Lewis 1997 Clin Exp Met 15:74).

- Monocytes and macrophages also infiltrate ischaemic lesions which are a feature of other
- 20 disease states including cerebral malaria (Kato *et al* 1996 Brain Res 734: 203-212; Patnaik *et al* 1994 Am J Trop Med Hyg 51: 642-647; Sakurai *et al* 1995 J Cardiol 26: 139-147); coronary heart disease (Stary *et al* 1994 J Arterio Thromb 14: 840-856; Ueda *et al* 1997 Hiroshima J. Med Sci 46: 31-42); and rheumatoid arthritis (Mapp *et al* 1995 Br Med Bull 51: 419-436; Sack *et al* 1994 Rheumatol Int 13: 181-186; Liote *et al* 1996 Clin Exp
- 25 Immunol 106: 13-19).

While monocytes and their differentiated derivatives, macrophages, are relatively long-lived cells *in vivo* a drawback associated with their use is their very limited proliferative potential.

- 30 Macrophages do not persist for long enough in the body for therapeutic genes introduced into them to provide prophylaxis throughout the potential lifetime of the individual. Thus

any therapy which depends on gene transfer to such cells will inevitably have a duration of action which is limited by the life-span of the recipient cells.

5 Although methods have been described which exploit macrophages as a delivery vehicle for marker and therapeutic genes to solid tumours there is a need to provide ways of exploiting HSCs to deliver NOIs to sites such as solid tumours which are characterised by ischaemia, such as hypoxia or low glucose concentration.

10 Furthermore there is a need to provide prophylactic vaccination for cancer and other related disorders which are characterised by ischaemia, such as hypoxia and low glucose concentration.

According to a first aspect of the present invention there is provided a modified haematopoietic stem cell (MHSC) comprising at least one expressable nucleotide sequence of interest (NOI) wherein the or each NOI is operably linked to one or more ischaemia like  
15 response element (ILRE).

According to a second aspect of the present invention there is provided a MHSC according to the present invention in combination with one or more agents that are capable of differentiating the MHSC.

20 According to a third aspect of the present invention there is provided a pharmaceutical composition comprising a MHSC according to the present invention optionally admixed with a pharmaceutically acceptable diluent, excipient or carrier.

25 According to a fourth aspect of the present invention there is provided a MHSC according to the present invention for use in medicine.

According to a fifth aspect of the present invention there is provided a method of expressing one or more NOIs in an ischaemic environment comprising expressing the one  
30 or more NOIs of the MHSC according to the present invention in the ischaemic environment.

According to a sixth aspect of the present invention there is provided the use of a MHSC according to the present invention in the manufacture of a medicament to treat a condition associated with or caused by ischaemia.

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According to a seventh aspect of the present invention there is provided a process of treating an individual in need of same comprising administering a MHSC according to the present invention, or a pharmaceutical composition according to the present invention, and allowing expression of one or more of the one or more NOIs.

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According to an eight aspect of the invention there is provided a modified cell comprising an element that is active in that cell; and an NOI; wherein the modified cell is prepared by transforming a cell by viral transduction with one or more viral vectors wherein at least one of which comprises the NOI.

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According to a ninth aspect of the present invention there is provided a hybrid viral vector system for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which  
20 secondary vector is capable of transducing a secondary target cell, wherein the primary viral vector and/or the secondary viral vector comprises an ILRE of the present invention or a cell specific regulatory element according to the present invention.

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According to a tenth aspect of the present invention there is provided a hybrid viral vector system wherein the primary vector is obtainable from or is based on a adenoviral vector and/or the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector, wherein the primary viral vector and/or the secondary viral vector comprises an ILRE of the present invention or a cell specific regulatory element according to the present invention.

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According to a eleventh aspect of the present invention there is provided one or more novel vectors or constructs or promoters or regulatory elements as defined herein.

5 According to a twelfth aspect of the present invention there is provided adenoviral vector constructs that show low basal activity in normal tissue but are strongly induced under ischaemic conditions.

10 According to a thirteenth aspect of the present invention there is provided lentiviral vector constructs that show low basal activity in normal tissue but are strongly induced under ischaemic conditions.

15 According to a fourteenth aspect of the present invention there is provided or more of a combination of adenoviral and lentiviral constructs that show low basal activity in normal tissue but are strongly induced under ischaemic conditions.

According to a fifteenth aspect of the present invention there is provided the use of an adenoviral vector to deliver an ILRE regulated gene to any cell type for use in any disease.

20 According to a sixteenth aspect of the present invention there is provided the use of a retroviral vector to deliver an ILRE regulated gene to any cell type for use in any disease.

25 According to a seventeenth aspect of the present invention there is provided the use of a combination of adenoviral and lentiviral vector to deliver an ILRE regulated gene to any cell type for use in any disease.

Preferably the ILRE is used in combination with a transcriptional regulatory element (such as a promoter), which transcriptional regulatory element is preferably active in one or more selected cell type(s), preferably being only active in one cell type.

30 This combination aspect of the present invention is called a responsive element, wherein the responsive element comprises at least the ILRE as herein defined.

Non-limiting examples of such a responsive element are presented as OBHRE1 and XiaMac. Another non-limiting example includes the ILRE in use in conjunction with an MLV promoter and/or a tissue restricted ischaemic responsive promoter.

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Preferably the ischaemic responsive promoter is a tissue restricted ischaemic responsive promoter.

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Preferably the tissue restricted ischaemic responsive promoter is a macrophage specific promoter restricted by repression.

Preferably the tissue restricted ischaemic responsive promoter is an endothelium specific promoter.

15

Preferably the vector is an ILRE regulated retroviral vector.

Preferably the vector is an ILRE regulated lentiviral vector.

Preferably the vector is an ILRE regulated adenoviral vector.

20

Preferably the vector is an ILRE regulated hybrid adenoviral /lentiviral vector.

Preferably the vector is an autoregulated hypoxia responsive lentiviral vector.

25

The term "ischaemia like response element" - otherwise written as ILRE - includes an element that is responsive to or is active under conditions of ischaemia or conditions that are like ischaemia or are caused by ischaemia. By way of example, conditions that are like ischaemia or are caused by ischaemia include hypoxia and/or low glucose concentration(s).



Ischaemia can be an insufficient supply of blood to a specific organ or tissue. A consequence of decreased blood supply is an inadequate supply of oxygen to the organ or tissue (hypoxia). Prolonged hypoxia may result in injury to the affected organ or tissue.

- 5 A preferred ILRE is an hypoxia response element (HRE).

The MHSC of the present invention may be prepared by use of a viral vector - which vector in turn may be delivered to a HSC by viral and/or non-viral means.

- 10 A example of a suitable agent that is capable of differentiating the MHSC is a cytokine and/or growth factor. In this embodiment, the MHSC may then differentiate into one or more different cell types.

- 15 In some applications it may be desirable to isolate the MHSC - such as prior to delivery to an individual. The MHSC may be isolated by standard techniques such filtration, centrifugation, micro-pipette.

- 20 An advantage of the present invention is that it provides means and methods for use in the treatment or prevention of conditions characterised by ischaemia, hypoxia or low glucose such as, without limitation, cancer, cerebral malaria, ischaemic heart disease or rheumatoid arthritis.

- 25 In one aspect, the present invention concerns the use of delivery systems to deliver NOIs to HSCs and more particularly to CD34<sup>+</sup> HSCs.

- 30 In another aspect, the invention provides a method of genetically engineering a HSC to contain at least one NOI, which method comprises transfecting or transducing a population of HSCs with a vector comprising at least one NOI, wherein the NOI is selected for treatment or prophylaxis of a condition characterised by ischaemia, hypoxia or low glucose.

In another aspect, the invention provides MHSCs produced by the above-mentioned method according to the invention.

5 In yet another aspect, the invention provides vectors suitable for use in the above-mentioned method, comprising at least one NOI and/or having at least one insertion site into which the NOI can be inserted. Such vectors are adapted to deliver at least one NOI to a HSC.

10 In a further aspect, the invention provides a medicament for treatment or prophylaxis of conditions characterised by ischaemia, hypoxia or low glucose, comprising a vector and/or MHSC as described together with a suitable pharmaceutically acceptable carrier.

15 In a still further aspect the invention provides a method for delivering at least one NOI to a population of HSCs from an individual to be treated, which method comprises contacting the cells with a vector as described under conditions to allow transfection or transduction of the cells; and reintroducing the transfected or transduced cells back into the individual.

20 The invention further provides a method for delivering at least one NOI to a population of HSCs of an individual to be treated, which method comprises administering to the individual a medicament as described herein.

25 Further provided according to the invention is a method of treatment or prophylaxis of cancer in a mammal, which method comprises isolating a population of cells enriched in HSCs from an individual to be treated, contacting the cells with a vector as described herein containing at least one NOI, under conditions to allow transfection or transduction of the cells, culturing the resulting engineered MHSCs under suitable conditions and reintroducing the cultured MHSCs or their differentiated progeny into the individual.

30 In accordance with the present invention, the NOI or NOIs can be any suitable nucleotide sequence. For example, each sequence can be independently DNA or RNA - which may be synthetically prepared or may be prepared by use of recombinant DNA techniques or

may be isolated from natural sources or may be combinations thereof. The sequence may be a sense sequence or an antisense sequence. There may be a plurality of sequences, which may be directly or indirectly joined to each other, or combinations thereof.

- 5 In one preferred embodiment, the present invention is based on the surprising use of a retroviral vector to transform one or more HSCs with one or more NOI(s) - wherein the transformed MHSC(s) can be used in a specific manner.

10 The retroviral vector aspect of the present invention may be derived from or may be derivable from any suitable retrovirus. Any of the following teachings are applicable to the present invention.

By way of background information, retroviruses are RNA viruses with a life cycle different to that of lytic viruses. In this regard, a retrovirus is an infectious entity that replicates  
15 through a DNA intermediate. When a retrovirus infects a cell, its genome is converted to a DNA form by a reverse transcriptase enzyme. The DNA copy serves as a template for the production of new RNA genomes and virally encoded proteins necessary for the assembly of infectious viral particles.

- 20 There are many retroviruses and examples include: murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV),  
25 Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV). A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

Details on the genomic structure of some retroviruses may be found in the art. By way of  
30 example, details on HIV may be found from the NCBI Genbank (i.e. Genome Accession No. AF033819).

Essentially, all wild type retroviruses contain three major coding domains, *gag*, *pol*, *env*, which code for essential virion proteins. Nevertheless, retroviruses may be broadly divided into two categories: namely, "simple" and "complex". These categories are distinguishable  
5 by the organisation of their genomes. Simple retroviruses usually carry only elementary information. In contrast, complex retroviruses also code for additional regulatory proteins derived from multiple spliced messages.

Retroviruses may even be further divided into seven groups. Five of these groups  
10 represent retroviruses with oncogenic potential. The remaining two groups are the lentiviruses and the spumaviruses. A review of these retroviruses is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 1-25).

15 All oncogenic members except the human T-cell leukemia virus-bovine leukemia virus group (HTLV-BLV) are simple retroviruses. HTLV, BLV and the lentiviruses and spumaviruses are complex. Some of the best studied oncogenic retroviruses are Rous sarcoma virus (RSV), mouse mammary tumour virus (MMTV) and murine leukemia virus (MLV) and the human T-cell leukemia virus (HTLV).

20 The lentivirus group can be split even further into "primate" and "non-primate". Examples of primate lentiviruses include the human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype  
25 "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

A distinction between the lentivirus family and other types of retroviruses is that  
30 lentiviruses have the capability to infect both dividing and non-dividing cells (Lewis *et al* 1992 EMBO. J 11: 3053-3058; Lewis and Emerman 1994 J. Virol. 68: 510-516). In

As shown in Figure 3, the basic molecular organisation of an infectious retroviral RNA genome is (5') R - U5 - *gag*, *pol*, *env* - U3-R (3'). In a defective retroviral vector genome *gag*, *pol* and *env* may be absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.

Reverse transcription of the virion RNA into double stranded DNA takes place in the cytoplasm and involves two jumps of the reverse transcriptase from the 5' terminus to the 3' terminus of the template molecule. The result of these jumps is a duplication of sequences located at the 5' and 3' ends of the virion RNA. These sequences then occur fused in tandem on both ends of the viral DNA, forming the long terminal repeats (LTRs) which comprise R U5 and U3 regions. On completion of the reverse transcription, the viral DNA is translocated into the nucleus where the linear copy of the retroviral genome, called a preintegration complex (PIC), is randomly inserted into chromosomal DNA with the aid of the virion integrase to form a stable provirus. The number of possible sites of integration into the host cellular genome is very large and very widely distributed.

The control of proviral transcription remains largely with the noncoding sequences of the viral LTR. The site of transcription initiation is at the boundary between U3 and R in the left hand side LTR (as shown above) and the site of poly (A) addition (termination) is at the boundary between R and U5 in the right hand side LTR (as shown above). U3 contains most of the transcriptional control elements of the provirus, which include the promoter and multiple enhancer sequences responsive to cellular and in some cases, viral transcriptional activator proteins. Some retroviruses have any one or more of the following genes such as *tat*, *rev*, *tax* and *rex* that code for proteins that are involved in the regulation of gene expression.

Transcription of proviral DNA recreates the full length viral RNA genomic and subgenomic-sized RNA molecules that are generated by RNA processing. Typically, all RNA products serve as templates for the production of viral proteins. The expression of

the RNA products is achieved by a combination of RNA transcript splicing and ribosomal frameshifting during translation.

5 RNA splicing is the process by which intervening or "intronic" RNA sequences are removed and the remaining "exonic" sequences are ligated to provide continuous reading frames for translation. The primary transcript of retroviral DNA is modified in several ways and closely resembles a cellular mRNA. However, unlike most cellular mRNAs, in which all introns are efficiently spliced, newly synthesised retroviral RNA must be diverted into two populations. One population remains unspliced to serve as the genomic  
10 RNA and the other population is spliced to provide subgenomic RNA.

The full-length unspliced retroviral RNA transcripts serve two functions: (i) they encode the *gag* and *pol* gene products and (ii) they are packaged into progeny virion particles as genomic RNA. Sub-genomic-sized RNA molecules provide mRNA for the remainder of  
15 the viral gene products. All spliced retroviral transcripts bear the first exon, which spans the U5 region of the 5' LTR. The final exon always retains the U3 and R regions encoded by the 3' LTR although it varies in size. The composition of the remainder of the RNA structure depends on the number of splicing events and the choice of alternative splice sites.

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In simple retroviruses, one population of newly synthesised retroviral RNA remains unspliced to serve as the genomic RNA and as mRNA for *gag* and *pol*. The other population is spliced, fusing the 5' portion of the genomic RNA to the downstream genes, most commonly *env*. Splicing is achieved with the use of a splice donor positioned  
25 upstream of *gag* and a splice acceptor near the 3' terminus of *pol*. The intron between the splice donor and splice acceptor that is removed by splicing contains the *gag* and *pol* genes. This splicing event creates the mRNA for envelope (Env) protein. Typically the splice donor is upstream of *gag* but in some viruses, such as ASLV, the splice donor is positioned a few codons into the *gag* gene resulting in a primary Env translation product  
30 that includes a few amino-terminal amino acid residues in common with Gag. The Env

protein is synthesised on membrane-bound polyribosomes and transported by the cell's vesicular traffic to the plasma membrane, where it is incorporated into viral particles.

Complex retroviruses generate both singly and multiply spliced transcripts that encode not only the *env* gene products but also the sets of regulatory and accessory proteins unique to these viruses. Complex retroviruses such as the lentiviruses, and especially HIV, provide striking examples of the complexity of alternative splicing that can occur during retroviral infection. For example, it is now known that HIV-1 is capable of producing over 30 different mRNAs by sub-optimal splicing from primary genomic transcripts. This selection process appears to be regulated as mutations that disrupt competing splice acceptors can cause shifts in the splicing patterns and can affect viral infectivity (Purcell and Martin 1993 J Virol 67: 6365-6378).

The relative proportions of full-length RNA and subgenomic-sized RNAs vary in infected cells and modulation of the levels of these transcripts is a potential control step during retroviral gene expression. For retroviral gene expression, both unspliced and spliced RNAs must be transported to the cytoplasm and the proper ratio of spliced and unspliced RNA must be maintained for efficient retroviral gene expression. Different classes of retroviruses have evolved distinct solutions to this problem. The simple retroviruses, which use only full-length and singly spliced RNAs regulate the cytoplasmic ratios of these species either by the use of variably efficient splice sites or by the incorporation of several *cis*-acting elements, that have been only partially defined, into their genome. The complex retroviruses, which direct the synthesis of both singly and multiply spliced RNA, regulate the transport and possibly splicing of the different genomic and subgenomic-sized RNA species through the interaction of sequences on the RNA with the protein product of one of the accessory genes, such as *rev* in HIV-1 and *rex* in HTLV-1.

With regard to the structural genes *gag*, *pol* and *env* themselves and in slightly more detail, *gag* encodes the internal structural protein of the virus. *Gag* is proteolytically processed into the mature proteins MA (matrix), CA (capsid) and NC (nucleocapsid). The *pol* gene encodes the reverse transcriptase (RT), which contains both DNA polymerase, and

associated RNase H activities and integrase (IN), which mediates replication of the genome. The *env* gene encodes the surface (SU) glycoprotein and the transmembrane (TM) protein of the virion, which form a complex that interacts specifically with cellular receptor proteins. This interaction leads ultimately to fusion of the viral membrane with  
5 the cell membrane.

The Env protein is a viral protein which coats the viral particle and plays an essential role in permitting viral entry into a target cell. The envelope glycoprotein complex of retroviruses includes two polypeptides: an external, glycosylated hydrophilic polypeptide  
10 (SU) and a membrane-spanning protein (TM). Together, these form an oligomeric "knob" or "knobbed spike" on the surface of a virion. Both polypeptides are encoded by the *env* gene and are synthesised in the form of a polyprotein precursor that is proteolytically cleaved during its transport to the cell surface. Although uncleaved Env proteins are able to bind to the receptor, the cleavage event itself is necessary to activate the fusion potential  
15 of the protein, which is necessary for entry of the virus into the host cell. Typically, both SU and TM proteins are glycosylated at multiple sites. However, in some viruses, exemplified by MLV, TM is not glycosylated.

Although the SU and TM proteins are not always required for the assembly of enveloped  
20 virion particles as such, they play an essential role in the entry process. In this regard, the SU domain binds to a receptor molecule, often a specific receptor molecule, on the target cell. It is believed that this binding event activates the membrane fusion-inducing potential of the TM protein after which the viral and cell membranes fuse. In some viruses, notably MLV, a cleavage event, resulting in the removal of a short portion of the cytoplasmic tail  
25 of TM, is thought to play a key role in uncovering the full fusion activity of the protein (Brody *et al* 1994 J Virol 68: 4620-4627; Rein *et al* 1994 J Virol 68: 1773-1781). This cytoplasmic "tail", distal to the membrane-spanning segment of TM remains on the internal side of the viral membrane and it varies considerably in length in different retroviruses.



Thus, the specificity of the SU/receptor interaction can define the host range and tissue tropism of a retrovirus. In some cases, this specificity may restrict the transduction potential of a recombinant retroviral vector. Here, transduction includes a process of using a viral vector to deliver a non-viral gene to a target cell. For this reason, many gene therapy experiments have used MLV. A particular MLV that has an envelope protein called 4070A is known as an amphotropic virus, and this can also infect human cells because its envelope protein "docks" with a phosphate transport protein that is conserved between man and mouse. This transporter is ubiquitous and so these viruses are capable of infecting many cell types. In some cases however, it may be beneficial, especially from a safety point of view, to specifically target restricted cells. To this end, several groups have engineered a mouse ecotropic retrovirus, which unlike its amphotropic relative normally only infects mouse cells, to specifically infect particular human cells. Replacement of a fragment of an Env protein with an erythropoietin segment produced a recombinant retrovirus which then binds specifically to human cells that express the erythropoietin receptor on their surface, such as red blood cell precursors (Maulik and Patel 1997 "Molecular Biotechnology: Therapeutic Applications and Strategies" 1997 Wiley-Liss Inc. pp 45).

Replacement of the *env* gene with a heterologous *env* gene is an example of a technique or strategy called pseudotyping. Pseudotyping is not a new phenomenon and examples may be found in WO-A-98/05759, WO-A-98/05754, WO-A-97/17457, WO-A-96/09400, WO-A-91/00047 and Mebatsion *et al* 1997 Cell 90, 841-847.

In addition to *gag*, *pol* and *env*, the complex retroviruses also contain "accessory" genes which code for accessory or auxillary proteins. Accessory or auxillary proteins are defined as those proteins encoded by the accessory genes in addition to those encoded by the usual replicative or structural genes, *gag*, *pol* and *env*. These accessory proteins are distinct from those involved in the regulation of gene expression, like those encoded by *tat*, *rev*, *tax* and *rex*. Examples of accessory genes include one or more of *vif*, *vpr*, *vpx*, *vpu* and *nef*. These accessory genes can be found in, for example, HIV (see, for example pages 802 and 803 of "Retroviruses" Ed. Coffin *et al* Pub. CSHL 1997). Non-essential accessory proteins

may function in specialised cell types, providing functions that are at least in part duplicative of a function provided by a cellular protein. Typically, the accessory genes are located between *pol* and *env*, just downstream from *env* including the U3 region of the LTR or overlapping portions of the *env* and each other.

5

The complex retroviruses have evolved regulatory mechanisms that employ virally encoded transcriptional activators as well as cellular transcriptional factors. These *trans*-acting viral proteins serve as activators of RNA transcription directed by the LTRs. The transcriptional *trans*-activators of the lentiviruses are encoded by the viral *tat* genes. Tat  
10 binds to a stable, stem-loop, RNA secondary structure, referred to as TAR, one function of which is to apparently optimally position Tat to *trans*-activate transcription.

In the general sense, retroviruses have been proposed as a delivery system (otherwise expressed as a delivery vehicle or delivery vector) for *inter alia* the transfer of a NOI, or a  
15 plurality of NOIs, to one or more sites of interest. The transfer can occur *in vitro*, *ex vivo*, *in vivo*, or combinations thereof. When used in this fashion, the retroviruses are typically called retroviral vectors or recombinant retroviral vectors. Retroviral vectors have even been exploited to study various aspects of the retrovirus life cycle, including receptor usage, reverse transcription and RNA packaging (reviewed by Miller, 1992 Curr Top  
20 Microbiol Immunol 158:1-24).

In a typical recombinant retroviral vector for use in gene therapy, at least part of one or more of the *gag*, *pol* and *env* protein coding regions may be removed from the virus. This makes the retroviral vector replication-defective. The removed portions may even be  
25 replaced by a NOI in order to generate a virus capable of integrating its genome into a host genome but wherein the modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in, for example, a therapeutic and/or a diagnostic effect. Thus, the transfer of a NOI into a site of interest is typically achieved by: integrating the NOI into the  
30 recombinant viral vector; packaging the modified viral vector into a virion coat; and

allowing transduction of a site of interest - such as a targeted cell or a targeted cell population.

5 It is possible to propagate and isolate quantities of retroviral vectors (e.g. to prepare suitable titres of the retroviral vector) for subsequent transduction of, for example, a site of interest by using a combination of a packaging or helper cell line and a recombinant vector.

10 In some instances, propagation and isolation may entail isolation of the retroviral *gag*, *pol* and *env* genes and their separate introduction into a host cell to produce a "packaging cell line". The packaging cell line produces the proteins required for packaging retroviral DNA but it cannot bring about encapsidation due to the lack of a *psi* region. However, when a recombinant vector carrying a NOI and a *psi* region is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant vector to produce the recombinant virus stock. This can be used to transduce cells to introduce the NOI into the  
15 genome of the cells. The recombinant virus whose genome lacks all genes required to make viral proteins can transduce only once and cannot propagate. These viral vectors which are only capable of a single round of transduction of target cells are known as replication defective vectors. Hence, the NOI is introduced into the host/target cell genome without the generation of potentially harmful retrovirus. A summary of the available  
20 packaging lines is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 449).

The design of retroviral packaging cell lines has evolved to address the problem of *inter alia* the spontaneous production of helper virus that was frequently encountered with early  
25 designs. As recombination is greatly facilitated by homology, reducing or eliminating homology between the genomes of the vector and the helper has reduced the problem of helper virus production. More recently, packaging cells have been developed in which the *gag*, *pol* and *env* viral coding regions are carried on separate expression plasmids that are independently transfected into a packaging cell line so that three recombinant events are  
30 required for wild type viral production. This reduces the potential for production of a

replication-competent virus. This strategy is sometimes referred to as the three plasmid transfection method (Soneoka *et al* 1995 Nucl. Acids Res. 23: 628-633).

Transient transfection can also be used to measure vector production when vectors are being developed. In this regard, transient transfection avoids the longer time required to generate stable vector-producing cell lines and is used if the vector or retroviral packaging components are toxic to cells. Components typically used to generate retroviral vectors include a plasmid encoding the Gag/Pol proteins, a plasmid encoding the Env protein and a plasmid containing a NOI. Vector production involves transient transfection of one or more of these components into cells containing the other required components. If the vector encodes toxic genes or genes that interfere with the replication of the host cell, such as inhibitors of the cell cycle or genes that induce apoptosis, it may be difficult to generate stable vector-producing cell lines, but transient transfection can be used to produce the vector before the cells die. Also, cell lines have been developed using transient infection that produce vector titre levels that are comparable to the levels obtained from stable vector-producing cell lines (Pear *et al* 1993, Proc Natl Acad Sci 90:8392-8396).

In view of the toxicity of some HIV proteins - which can make it difficult to generate stable HIV-based packaging cells - HIV vectors are usually made by transient transfection of vector and helper virus. Some workers have even replaced the HIV Env protein with that of vesicular stomatis virus (VSV). Insertion of the Env protein of VSV facilitates vector concentration as HIV/VSV-G vectors with titres of  $5 \times 10^5$  ( $10^8$  after concentration) have been generated by transient transfection (Naldini *et al* 1996 Science 272: 263-267). Thus, transient transfection of HIV vectors may provide a useful strategy for the generation of high titre vectors (Yee *et al* 1994 PNAS. 91: 9564-9568).

With regard to vector titre, the practical uses of retroviral vectors have been limited largely by the titres of transducing particles which can be attained in *in vitro* culture (typically not more than  $10^8$  particles/ml) and the sensitivity of many enveloped viruses to traditional biochemical and physicochemical techniques for concentrating and purifying viruses.

By way of example, several methods for concentration of retroviral vectors have been developed, including the use of centrifugation (Fekete and Cepko 1993 Mol Cell Biol 13: 2604-2613), hollow fibre filtration (Paul *et al* 1993 Hum Gene Ther 4: 609-615) and tangential flow filtration (Kotani *et al* 1994 Hum Gene Ther 5: 19-28). Although a 20-fold increase in viral titre can be achieved, the relative fragility of retroviral Env protein limits the ability to concentrate retroviral vectors and concentrating the virus usually results in a poor recovery of infectious virions. While this problem can be overcome by substitution of the retroviral Env protein with the more stable VSV-G protein, as described above, which allows for more effective vector concentration with better yields, it suffers from the drawback that the VSV-G protein is quite toxic to cells.

Although helper-virus free vector titres of  $10^7$  cfu/ml are obtainable with currently available vectors, experiments can often be done with much lower-titre vector stocks. However, for practical reasons, high-titre virus is desirable, especially when a large number of cells must be infected. In addition, high titres are a requirement for transduction of a large percentage of certain cell types. For example, the frequency of human hematopoietic progenitor cell infection is strongly dependent on vector titre, and useful frequencies of infection occur only with very high-titre stocks (Hock and Miller 1986 Nature 320: 275-277; Hogge and Humphries 1987 Blood 69: 611-617). In these cases, it is not sufficient simply to expose the cells to a larger volume of virus to compensate for a low virus titre. On the contrary, in some cases, the concentration of infectious vector virions may be critical to promote efficient transduction.

Workers are trying to create high titre vectors for use in gene delivery. By way of example, a comparison of different vector designs has proved useful in helping to define the essential elements required for high-titre viral production. Early work on different retroviral vector design showed that almost all of the internal protein-encoding regions of MLVs could be deleted without abolishing the infectivity of the vector (Miller *et al* 1983 Proc Natl Acad Sci 80: 4709-4713). These early vectors retained only a small portion of the 3' end of the *env*-coding region. Subsequent work has shown that all of the *env*-gene-coding sequences can be removed without further reduction in vector titre (Miller and Rosman 1989 Biotechnology 7: 980-990; Morgenstern and Land 1990 Nucleic Acids Res

18: 3587-3596). Only the viral LTRs and short regions adjoining the LTRs, including the segments needed for plus- and minus-strand DNA priming and a region required for selective packaging of viral RNA into virions (the *psi* site; Mann *et al* 1983 Cell 33: 153-159) were deemed necessary for vector transmission. Nevertheless, viral titres obtained with these early vectors were still about tenfold lower than the parental helper virus titre.

Additional experiments indicated that retention of sequences at the 5' end of the *gag* gene significantly raised viral vector titres and that this was due to an increase in the packaging efficiency of viral RNA into virions (Armentano *et al* 1987 J Virol 61: 1647-1650; Bender *et al* 1987 J Virol 61: 1639-1646; Adam and Miller 1988 J Virol 62: 3802-3806). This effect was not due to viral protein synthesis from the *gag* region of the vector because disruption of the *gag* reading frame or mutating the *gag* codon to a stop codon had no effect on vector titre (Bender *et al* 1987 *ibid*). These experiments demonstrated that the sequences required for efficient packaging of genomic RNA in MLV were larger than the *psi* signal previously defined by deletion analysis (Mann *et al* 1983 *ibid*). In order to obtain high titres ( $10^6$  to  $> 10^7$ ), it was shown to be important that this larger signal, called *psi* plus, be included in retroviral vectors. It has now been demonstrated that this signal spans from upstream of the splice donor to downstream of the *gag* start codon (Bender *et al* 1987 *ibid*). Because of this position, in spliced *env* expressing transcripts this signal is deleted. This ensures that only full length transcripts containing all three essential genes for viral life cycle are packaged.

In addition to manipulating the retroviral vector with a view to increasing vector titre, retroviral vectors have also been designed to induce the production of a specific NOI (usually a marker protein) in transduced cells. As already mentioned, the most common retroviral vector design involves the replacement of retroviral sequences with one or more NOIs to create replication-defective vectors. The simplest approach has been to use the promoter in the retroviral 5' LTR to control the expression of a cDNA encoding an NOI or to alter the enhancer/promoter of the LTR to provide tissue-specific expression or inducibility.

Alternatively, a single coding region has been expressed by using an internal promoter which permits more flexibility in promoter selection.

These strategies for expression of an NOI have been most easily implemented when the NOI is a selectable marker, as in the case of hypoxanthine-guanine phosphoribosyl transferase (*hprt*) (Miller *et al* 1983 Proc Natl Acad Sci 80: 4709-4713) which facilitates the selection of vector transduced cells. If the vector contains an NOI that is not a selectable marker, the vector can be introduced into packaging cells by co-transfection with a selectable marker present on a separate plasmid. This strategy has an appealing advantage for gene therapy in that a single protein is expressed in the ultimate target cells and possible toxicity or antigenicity of a selectable marker is avoided. However, when the inserted gene is not selectable, this approach has the disadvantage that it is more difficult to generate cells that produce a high titre vector stock. In addition it is usually more difficult to determine the titre of the vector.

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The current methodologies used to design retroviral vectors that express two or more proteins have relied on three general strategies. These include: (i) the expression of different proteins from alternatively spliced mRNAs transcribed from one promoter; (ii) the use of the promoter in the 5' LTR and internal promoters to drive transcription of different cDNAs and (iii) the use of internal ribosomal entry site (IRES) elements to allow translation of multiple coding regions from either a single mRNA or from fusion proteins that can then be expressed from an open reading frame.

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Vectors containing internal promoters have been widely used to express multiple genes. An internal promoter makes it possible to exploit the promoter/enhancer combinations other than the viral LTR for driving gene expression. Multiple internal promoters can be included in a retroviral vector and it has proved possible to express at least three different cDNAs each from its own promoter (Overell *et al* 1988 Mol Cell Biol 8: 1803-1808).

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A number of vectors have been developed based on various members of the lentivirus sub-family of the retroviridae and a number of these are the subject of patent applications

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- (PCT/GB97/02857; PCT US96/15406). The simplest vectors constructed from HIV-1 have the complete HIV genome except for a deletion of part of the env coding region or replacement of the nef coding region (Richardson, J. H., Child, L. A. & Lever, A. M. (1993) *J Virol* 67, 3997-4005., Buchschacher, G. L., Jr. & Panganiban, A. T. (1992) *J Virol* 66, 2731-9, Richardson, J. H., Kaye, J. F., Child, L. A. & Lever, A. M. (1995) *J Gen Virol* 76, 691-6, Kaye, J. F., Richardson, J. H. & Lever, A. M. (1995) *J Virol* 69, 6588-92, Carroll, R., Lin, J. T., Dacquel, E. J., Mosca, J. D., Burke, D. S. & St Louis, D. C. (1994) *J Virol* 68, 6047-51. A number of promoter /reporter cassettes have been inserted at these positions ADDIN ENRf8 ( Page, K. A., Landau, N. R. & Littman, D. R. (1990) *J Virol* 64, 5270-6., Shimada, T., Fujii, H., Mitsuya, H. & Nienhuis, A. W. (1991) *J Clin Invest* 88, 1043-76, 7). Notably these vectors express gag/pol and all of the accessory genes hence require only an envelope to produce infectious virus particles. Of the accessory genes vif, vpr, vpu and nef are non-essential, however it has been proposed that accessory gene expression does influence titre
- 15 (Fan, L. & Peden, K. (1992) *Virology* 190, 19-29, Gabuzda, D. H., Lever, A., Terwilliger, E. & Sodroski, J. (1992) *J Virol* 66, 3306-15, Schubert, U., Clouse, K. A. & Strebel, K. (1995) *J Virol* 69, 7699-711, Miller, M. D., Warmerdam, M. T., Gaston, I., Greene, W. C. & Feinberg, M. B. (1994) *J Exp Med* 179, 101-13 Zufferey, R., Nagy, D., Mandel, R.J., Naldini, L. & Trono, (1997) *Nature Biotechnology* 15, 871-875, Takahashi, K., Wesselingh, S. L., Griffin, D.E., McArthur, J. C. Johnson, R.T. & Glass, J. D. (1996) *Ann. Neurol.* 39, 705-711. More recently however vector have been describe that are efficient yet lack most or all of the accessory factors Poeschla, E., Corbeau, P. & Wong-Staal, F. (1996) *Proc Natl Acad Sci U S A* 93, 11395-9. Naldini, L., Blomer, U., Gage, F. H., Trono, D. & Verma, I. M. (1996) *Proc Natl Acad Sci U S A* 93, 11382-8. Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M. & Trono, D. (1996) *Science* 272, 263-7, Blomer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. & Gage, F. (1997) *Journal of Virology* 71, 6641-6649, Kim, V.N., Mitrophanous, K., Kingsman, S.M. and Kingsman, A.J. 1998. *J. Virol.*, 72, 811-816.
- 25
- 30 The general format for these lentiviral vectors is, HIV-1 5'LTR and leader, some gag coding region sequences (to supply packaging functions), a reporter cassette, the rev



response element (RRE) and the 3'LTR. In these vectors gag/pol, accessory gene products and envelope functions are supplied either from a single plasmid or from two or more co-transfected plasmids, or by co-infection of vector containing cells with HIV. Transactivation has also been utilised in an HIV-based vector in which the U3 region of the 5'LTR is altered to contain two copies of the tax responsive element (TRE), which allows transactivation by the tax protein of HTLV-I. This is proposed as a therapeutic for adult T-cell leukemia/lymphoma (Lin, H. C., Bodkin, M., Lal, R. B. & Rabson, A. B. (1995) J Virol 69, 7216-25.) Vectors have also been constructed that can be expressed using a constitutive promoter such as the CMV promoter (e.g Kim et al 1998 *ibid*) More recently the lentiviral vector configurations have been further refined. For example self inactivating HIV vectors have been produced where the HIV LTR is deleted to restrict expression to the internal cassette (Myoshi et al 1998 J. Virol 72, 8150)

Where co-infection of a target cell with HIV occurs the vector can be spread throughout a culture of HIV permissive cells. This principal has been used for the development of vectors for the treatment or limitation of HIV-1 infection (Dropulic, B., Hermankova, M. & Pitha, P. M. (1996) Proc Natl Acad Sci U S A 93, 11103-8., Kim, J. H., McLinden, R. J., Mosca, J. D., Vahey, M. T., Greene, W. C. & Redfield, R. R. (1996) J Acquir Immune Defic Syndr Hum Retrovirol 12, 343-51, Poeschla et al 1996 PNAS 93, 11395.

Numerous studies have now described the utility of HIV based vectors for gene transfer to non-dividing cells. (e.g. retina, Myoshi et al 1997, PNAS 94, 10319-23., neurons, Blomer et al 1997 J. Virol 71, 6641, Blomer et al 1998 95, 2603, liver, muscle, Kafri et al Nature Genetics 1997 17, 314). Other lentiviral vectors have also been developed for example ELAV (GB 9727135.7) and FIV (Poeschla et al 1998, Nature Medicine, 4, 354) and Maedi-Visna (International Application Number: PCT/GB95/00663. Priority date: 9 Dec 1994/24 March 1995 Packaging-Deficient Lentiviruses Lever, A.M.L., Harrison, G.P., Hunter, E).

Retroviral gene transfer into murine HSCs in the general sense has already been reported (Williams *et al* Nature 1984 310: 476-479). Here, the expression of a full-length MDRI

(multidrug resistance) cDNA gene in murine HSCs was shown to render them resistant to various anticancer drugs. Similarly, murine haematopoietic progenitor cells in bone marrow or peripheral blood cells have been shown to be protected from the toxicity of anticancer chemotherapy by retroviral transduction of the MDRI gene (Licht *et al* 1995 Cytokines Mol Ther 1: 11-20). Sustained human hematopoiesis in immunodeficient mice has also been demonstrated by cotransplantation of CD34<sup>+</sup> progenitor cells which had been transduced *in vitro* with a recombinant retroviral vector comprising either a neomycin phosphotransferase gene (*neo*) or a human glucocerebrosidase cDNA (Nolta *et al* 1994 Blood 83: 3041-3051). Murine HSCs have also been transduced with a two gene retroviral vector containing a reporter (*LacZ*) and selectable marker (*neo*). *LacZ* expression was detectable in the PBL of the recipients (Asami *et al* 1996 Eur J Haematol 57: 278-285).

*In vivo* murine studies have indicated that the pretreatment of donor mice with 5-fluorouracil prior to harvest of bone marrow can improve transduction efficiencies by inducing the cycling of primitive cells and increasing the susceptibility to retroviral infection and integration. The co-culture of target cells with retroviral producer cell line and the use of cell lines capable of producing at least 10<sup>5</sup> viral particles per ml has also improved efficiencies (Bodine *et al* 1991 Exp Hematol 19: 206-212). Successful gene transfer into long terms re-populating cells has been achieved in virtually all recipient mice with reconstitution of multiple haematopoietic lineages stably with 1-50% or more cells carrying the proviral genome (Fraser *et al* 1990 Blood 76: 1071-1076).

Retroviral gene transfer into human HSCs in the general sense has been reported (Duphar and Emmons 1994 Stem Cells 12: 563-576). Also, committed human progenitor cells such as colony forming units-granulocyte macrophage (CFU-GM) or burst forming units-erythroid (BFU-E) have been transduced by retroviral vectors at very high efficiencies (often greater than 50%) in the presence of various combinations of growth factors such as IL-3, IL-6 and Stem Cell Factor (SCF) or in the presence of primary marrow stroma or the retroviral producer cell line (Nolta *et al* 1990 Hum Gen Ther 1: 257-268; Moore *et al* 1992 Blood 79: 1393-1399; Cournoyer *et al* 1991 Hum Gen Ther 2: 203-213).

The presence of specific extracellular matrix components such as fibronectin may be of some importance as well (Moritz *et al* J Clin Invest 1994 93: 1451-1457). More primitive human long-term culture initiating cells (LTC-IC) have been transduced at equivalent efficiencies under similiar transduction conditions (Moore *et al* 1992 *ibid*; Hughes *et al* 5 1989 Blood 74: 1915-1922; Hughes *et al* 1992 J Clin Invest 89: 1817-1824). The expression of high levels of a human tumour antigen, epithelial cell mucin (MUC-1) on human dendritic cells (DCs) has been achieved by retroviral transduction of CD34<sup>+</sup> progenitor cells and their subsequent cytokine-induced differentiation into Dcs (Henderson *et al* 1996 Cancer Res 56: 3763-3770).

10 Despite the general teachings in the art that retroviral systems have been used to transfer genes into HSCs, the prior art does not teach or suggest the present invention wherein one or more NOIs are used to transform one or more HSCs and in a specific manner.

In accordance with the present invention, suitable NOI sequences include those that are of 15 therapeutic and/or diagnostic application such as, but are not limited to: sequences encoding cytokines, chemokines, hormones, antibodies, engineered immunoglobulin-like molecules, a single chain antibody, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a tumour suppressor 20 protein and growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives thereof (such as with an associated reporter group). When included, such coding sequences may be typically operatively linked to a suitable promoter, which may be a promoter driving expression of a ribozyme(s), or a different promoter or promoters, such as in one or more specific cell types.

25 Suitable NOIs for use in the invention in the treatment or prophylaxis of cancer include NOIs encoding proteins which: destroy the target cell (for example a ribosomal toxin), act as: tumour suppressors (such as wild-type p53); activators of anti-tumour immune mechanisms (such as cytokines, co-stimulatory molecules and immunoglobulins); 30 inhibitors of angiogenesis; or which provide enhanced drug sensitivity (such as pro-drug activation enzymes); indirectly stimulate destruction of target cell by natural effector cells

(for example, strong antigen to stimulate the immune system or convert a precursor substance to a toxic substance which destroys the target cell (for example a prodrug activating enzyme). Encoded proteins could also destroy bystander tumour cells (for example with secreted antitumour antibody-ribosomal toxin fusion protein), indirectly stimulated destruction of bystander tumour cells (for example cytokines to stimulate the immune system or procoagulant proteins causing local vascular occlusion) or convert a precursor substance to a toxic substance which destroys bystander tumour cells (eg an enzyme which activates a prodrug to a diffusible drug).

Also, the delivery of NOI(s) encoding antisense transcripts or ribozymes which interfere with expression of cellular genes for tumour persistence (for example against aberrant *myc* transcripts in Burkitts lymphoma or against *bcr-abl* transcripts in chronic myeloid leukemia. The use of combinations of such NOIs is also envisaged.

Instead of or as well as being selectively expressed in target tissues, the NOI or NOIs may encode a pro-drug activation enzyme or enzymes which have no significant effect or no deleterious effect until the individual is treated with one or more pro-drugs upon which the enzyme or enzymes act. In the presence of the active NOI, treatment of an individual with the appropriate pro-drug leads to enhanced reduction in tumour growth or survival.

20

A pro-drug activating enzyme may be delivered to a tumour site for the treatment of a cancer. In each case, a suitable pro-drug is used in the treatment of the patient in combination with the appropriate pro-drug activating enzyme. An appropriate pro-drug is administered in conjunction with the vector. Examples of pro-drugs include: etoposide phosphate (with alkaline phosphatase, Senter *et al* 1988 Proc Natl Acad Sci 85: 4842-4846); 5-fluorocytosine (with cytosine deaminase, Mullen *et al* 1994 Cancer Res 54: 1503-1506); Doxorubicin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase, Kerr *et al* 1990 Cancer Immunol Immunother 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with  $\beta$ -lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli *et al* 1988 Proc Natl Acad Sci 85: 7572-7576); mustard pro-

drugs with nitroreductase (Friedlos *et al* 1997 J Med Chem 40: 1270-1275) and Cyclophosphamide (with P450 Chen *et al* 1996 Cancer Res 56: 1331-1340).

5 Examples of suitable pro-drug activation enzymes for use in the invention include a thymidine phosphorylase which activates the 5-fluoro-uracil pro-drugs capcetabine and furtulon; thymidine kinase from Herpes Simplex Virus which activates ganciclovir; a cytochrome P450 which activates a pro-drug such as cyclophosphamide to a DNA damaging agent; and cytosine deaminase which activates 5-fluorocytosine. Preferably, an enzyme of human origin is used

10

Suitable NOIs for use in the treatment or prevention of ischaemic heart disease include NOIs encoding plasminogen activators. Suitable NOIs for the treatment or prevention of rheumatoid arthritis or cerebral malaria include genes encoding anti-inflammatory proteins, antibodies directed against tumour necrosis factor (TNF) alpha, and anti-adhesion  
15 molecules (such as antibody molecules or receptors specific for adhesion molecules).

Examples of hypoxia regulatable therapeutic NOIs can be found in PCT/GB95/00322 (WO-A-9521927).

20 The expression products encoded by the NOIs may be proteins which are secreted from the cell. Alternatively the NOI expression products are not secreted and are active within the cell. In either event, it is preferred for the NOI expression product to demonstrate a bystander effector or a distant bystander effect; that is the production of the expression product in one cell leading to the killing of additional, related cells, either neighbouring or  
25 distant (e.g. metastatic), which possess a common phenotype.

The vector may also contain one or more cytokine-encoding NOIs which serve to direct the subsequent differentiation of the MHSCs containing the vector. Suitable cytokines and growth factors include but are not limited to: ApoE, Apo-SAA, BDNF, Cardiotrophin-1,  
30 EGF, ENA-78, Eotaxin, Eotaxin-2, Exodus-2, FGF-acidic, FGF-basic, fibroblast growth factor-10 (Marshall 1998 Nature Biotechnology 16: 129).FLT3 ligand (Kimura *et al*.

(1997), Fractalkine (CX3C), GDNF, G-CSF, GM-CSF, GF- $\beta$ 1, insulin, IFN- $\gamma$ , IGF-I, IGF-II, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (72 a.a.), IL-8 (77 a.a.), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18 (IGIF), Inhibin  $\alpha$ , Inhibin  $\beta$ , IP-10, keratinocyte growth factor-2 (KGF-2), KGF, Leptin, LIF, Lymphotoxin, Mullerian inhibitory substance, monocyte colony inhibitory factor, monocyte attractant protein (Marshall 1998 *ibid*), M-CSF, MDC (67 a.a.), MDC (69 a.a.), MCP-1 (MCAF), MCP-2, MCP-3, MCP-4, MDC (67 a.a.), MDC (69 a.a.), MIG, MIP-1 $\alpha$ , MIP-1 $\beta$ , MIP-3 $\alpha$ , MIP-3 $\beta$ , MIP-4, myeloid progenitor inhibitor factor-1 (MPIF-1), NAP-2, Neurturin, Nerve growth factor,  $\beta$ -NGF, NT-3, NT-4, Oncostatin M, PDGF-AA, PDGF-AB, PDGF-BB, PF-4, RANTES, SDF1 $\alpha$ , SDF1 $\beta$ , SCF, SCGF, stem cell factor (SCF), TARC, TGF- $\alpha$ , TGF- $\beta$ , TGF- $\beta$ 2, TGF- $\beta$ 3, tumour necrosis factor (TNF), TNF- $\alpha$ , TNF- $\beta$ , TNIL-1, TPO, VEGF, GCP-2, GRO/MGSA, GRO- $\beta$ , GRO- $\gamma$ , HCC1, 1-309,

For some applications, a combination of some of these cytokines may be preferred, in particular a combination which includes IL-3, IL-6 and SCF, for the maintenance and expansion of stem cell populations. For differentiation *in vitro*, further cytokines may be added such as GM-CSF and M-CSF to induce differentiation of macrophages or GM-CSF and G-CSF to obtain neutrophils. On reintroduction of the engineered cells into the individual from which they were derived, the body's own mechanisms then permit the cells or their differentiated progeny to migrate into the affected area e.g. the tumour.

One or more of the NOIs may be fused. That is, the NOI coding sequence may encode a fusion protein or a segment of a coding sequence. For example, pIXY321 is a genetically engineered fusion protein of GM-CSF and IL-3 (Bhalla *et al* 1995 Leukemia 11: 1851-1856) which exhibits biologic effects of both its parent cytokines *in vitro* and in preclinical studies (Vadhan-Raj *et al* 1995 Blood 86: 2098-2105).

Optionally, another NOI may be a suicide gene, expression of which in the presence of an exogenous substance results in the destruction of the transfected or transduced cell. An example of a suicide gene includes the herpes-simplex virus thymidine kinase gene (HSV

*tk*) which can kill infected and bystander cells following treatment with ganciclovir (Robbins *et al* Tibtech 1998 16: 35-40).

Optionally another NOI may be a targeting protein (such as an antibody to the stem cell factor receptor (WO9217505; WO9221766). For example, recombinant (ecotropic) retroviruses displaying an antibody (or growth factor or peptide) against a receptor present on HSCs (CD34 or stem cell factor, for example) might be used for targeted cell delivery to these cells, either *ex vivo* by incubating unfractionated bone marrow with virus or by intravenous delivery of virus.

10

Ligands and antibodies may be utilised to target selected cell types, including for example, monoclonal antibody c-SF-25 to target a 125kD antigen on human lung carcinoma (Takahashi *et al* 1993 Science 259:1460); antibodies to various lung cancer antigens (Souhami 1992 Thorax 47: 53-56); antibodies to human ovarian cancer antigen 14C1 (Gallagher *et al* 1991 Br J Cancer 64: 35-40); antibodies to H/Lev/ILeb antigens to target lung carcinoma (Masayuki *et al* 1992 N Eng J Med 327:14-18); nerve growth factor to target nerve growth factor receptors on neural tumours (Chao *et al* 1986 Science 232: 518); the Fc receptor to target macrophages (Anderson and Looney 1987 Immunol Today 1: 264-266); lectins (Sharon and Lis 1989 Science 246: 227); collagen type I to target colon cancer (Pullam and Bodmer 1992 Nature 356: 529); Interleukin-1 to target the Interleukin-1 receptor on T Cells (Fanslow *et al* 1990 Science 248: 739); acetylated low density lipoproteins ("LDL") to target macrophage scavenger receptors (and atherosclerotic plaques; see Brown *et al* 1983 Ann Rev Biochem 52: 223-261); as well as other acetylated molecules which target macrophage scavenger receptors (Paulinski *et al* PNAS 86: 1372-1376); viral receptors (Haywood 1994 J Virol 68(1): 1-5); transferrin to target transferrin receptors on tumour cells (Huebers *et al* 1987 Physio Rev 67: 520-582); vasoendothelial growth factor ("vegF") to target cells where increased vascularisation occurs; and urokinase plasminogen activator receptor (UPAR).

Alternatively, ligands may be selected from libraries created utilising recombinant techniques (Scott and Smith 1990 Science 249: 386; Devlin *et al* 1990 Science 249: 404;

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Houghten *et al* 1991 Nature 354: 84; Matthews and Wells 1993 Science 260: 1113; Nisim *et al* 1994 EMBO J 13(3) 692-698) or equivalent techniques utilising organic compound libraries.

- 5 In addition to the therapeutic gene or genes and the expression regulatory elements described, the vector may contain additional genetic elements for the efficient or regulated expression of the gene or genes, including promoters/enhancers, translation initiation signals, internal ribosome entry sites (IRES), splicing and polyadenylation signals.
- 10 The NOI or NOIs may be under the expression control of an expression regulatory element, usually a promoter or a promoter and enhancer. The enhancer and/or promoter may be preferentially active in a hypoxic or ischaemic or low glucose environment, such that the NOI is preferentially expressed in the particular tissues of interest, such as in the environment of a tumour, arthritic joint or other sites of ischaemia. Thus any significant
- 15 biological effect or deleterious effect of the NOI on the individual being treated may be reduced or eliminated. The enhancer element or other elements conferring regulated expression may be present in multiple copies. Likewise, or in addition, the enhancer and/or promoter may be preferentially active in one or more specific cell types - such as any one or more of macrophages, endothelial cells or combinations thereof. Further
- 20 examples include include respiratory airway epithelial cells, hepatocytes, muscle cells, cardiac myocytes, synoviocytes, primary mammary epithelial cells and post-mitotically terminally differentiated non-replicating cells such as macrophages neurons.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase

25 binding site in the Jacob-Monod theory of gene expression.

The term "enhancer" includes a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.



The promoter and enhancer of the transcription units encoding the secondary vector are preferably strongly active, or capable of being strongly induced, in the primary target cells under conditions for production of the secondary viral vector. The promoter and/or enhancer may be constitutively efficient, or may be tissue or temporally restricted in their activity. Examples of suitable tissue restricted promoters/enhancers are those which are highly active in tumour cells such as a promoter/enhancer from a MUC1 gene, a CEA gene or a 5T4 antigen gene. Examples of temporally restricted promoters/enhancers are those which are responsive to ischaemia and/or hypoxia, such as hypoxia response elements or the promoter/enhancer of a grp78 or a grp94 gene. The alpha fetoprotein (AFP) promoter is also a tumour-specific promoter. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

Preferably the promoters of the present invention are tissue specific. That is, they are capable of driving transcription of a NOI or NOI(s) in one tissue while remaining largely "silent" in other tissue types.

The term "tissue specific" means a promoter which is not restricted in activity to a single tissue type but which nevertheless shows selectivity in that they may be active in one group of tissues and less active or silent in another group. A desirable characteristic of the promoters of the present invention is that they possess a relatively low activity in the absence of activated hypoxia-regulated enhancer elements, even in the target tissue. One means of achieving this is to use "silencer" elements which suppress the activity of a selected promoter in the absence of hypoxia.

The level of expression of an NOI or NOIs under the control of a particular promoter may be modulated by manipulating the promoter region. For example, different domains within a promoter region may possess different gene regulatory activities. The roles of these different regions are typically assessed using vector constructs having different variants of the promoter with specific regions deleted (that is, deletion analysis). This

approach may be used to identify, for example, the smallest region capable of conferring tissue specificity or the smallest region conferring hypoxia sensitivity.

5 A number of tissue specific promoters, described above, may be particularly advantageous in practising the present invention. In most instances, these promoters may be isolated as convenient restriction digestion fragments suitable for cloning in a selected vector. Alternatively, promoter fragments may be isolated using the polymerase chain reaction. Cloning of the amplified fragments may be facilitated by incorporating restriction sites at the 5' end of the primers.

10

Promoters suitable for cardiac-specific expression include the promoter from the murine cardiac  $\alpha$ -myosin heavy chain (MHC) gene. Suitable vascular endothelium-specific promoters include the Et-1 promoter and von Willebrand factor promoter.

15

Prostate specific promoters include the 5' flanking region of the human glandular kallikrein-1 (hKLK2) gene and the prostate specific antigen (hKLK3).

20

Examples of promoters/enhancers which are cell specific include a macrophage-specific promoter or enhancer, such as CSF-1 promoter-enhancer, or elements from a mannose receptor gene promoter-enhancer (Rouleux *et al* 1994 Exp Cell Res 214:113-119). Alternatively, promoter or enhancer elements which are preferentially active in neutrophils, or a lymphocyte-specific enhancer such as an IL-2 gene enhancer, may be used.

25

As indicated above, the present invention is based on the surprising finding that it is possible to transform one or more HSCs and for a specific purpose.

30

By way of background information, monocytes and their differentiated derivatives, macrophages are derived from a reservoir of embryonic cells, called HSCs which are capable of giving rise to a variety of distinct cell types. HSCs, in mammals, are found within the fetal liver, spleen and bone marrow but after birth and throughout adult life, they are normally found only in the bone marrow. HSCs differentiate into various cell lineages

under the influence of microenvironmental factors such as cell-to-cell interactions and the presence of soluble cell cytokines.

Four major cell lineages arise from the HSCs. These include: Erythroid (Erythrocytes);  
5 Megakaryocytic (platelets); Myeloid (granulocytes and mononuclear phagocytes); and  
Lymphoid (lymphocytes). In particular, the myeloid and lymphoid lineages are critical to  
the functioning of the immune system.

Myelopoiesis commences in the liver of the human foetus at about six weeks of gestation.  
10 Studies in which colonies have been grown *in vitro* from individual stem cells have shown  
that the first progenitor cell derived from HSCs is the colony forming unit (CFU) which  
can give rise to Granulocytes, Erythrocytes, Monocytes and Megakaryocytes (CFU-  
GEMM).  
15 Maturation of these cells occurs under the influence of a network of tissue specific protein  
regulators which have been given a variety of names including growth factors, cytokines  
and interleukins. In the main, there is no functional or structural characteristic that  
distinguishes the different classes of growth factors. Most factors appear to be capable of  
stimulating multiple biological responses that depend critically on the differentiation state  
20 of their target cells. For example, one of the haemopoietic growth factors, granulocyte  
colony stimulating factor (G-CSF) stimulates proliferation of immature bone marrow cells  
as well as activating bacterial killing by mature neutrophils. Erythropoietin (EPO) and  
thrombopoietin (TPO) are structurally similar cytokines and support respectively, the  
proliferation and differentiation for erythroid and megakaryocytic lineages as well as more  
25 primitive progenitors (Gotoh *et al* 1997 Ann Hematol 75: 207-213). TPO initiates its  
biologic effects by binding to the Mpl receptor, which is a member of the haematopoietic  
receptor family (Broudy *et al* 1997 Blood 89: 1896-1904). HOXB4 has been shown to be  
an important regulator of very early but not late haematopoietic cell proliferation  
(Sauvageau *et al* 1995 Genes Dev 9: 1753-1765). The soluble Kit ligand proteins (Kls) act  
30 as a ligand for the transmembrane tyrosine kinase receptor C-kit and stimulate mast cell  
and erythroid progenitors (91EP-810609). The interleukins, include IL-1, IL-2, IL-3, IL-4,

IL-5, IL-6 and IL-12, which are capable of activating HSCs (see "Molecular biology and Biotechnology Ed RA Meyers 1995 VCH Publishers Inc p 392-397).

5 These mediators, which are important in the positive regulation of haemopoiesis, are derived mainly from stromal cells in the bone marrow, but they are also produced by mature forms of differentiated myeloid and lymphoid cells. There are a number of successful growth factor combinations in use but combinations of IL-3 and IL-6 with or without other factors such as stem cell factor (SCF) active on primitive cells have achieved the best results (Bodine *et al* 1989 Proc Natl Acad Sci 86: 8897-8901; Luskey *et al* 1992 10 Blood 80: 396-402; Fraser *et al* 1990 Blood 76: 1071-1076). Other cytokines (such as TGF $\beta$ ) may downregulate haemapoiesis.

The CFU-GM cell is the precursor of both neutrophils and mononuclear phagocytes. As the CFU-GM differentiates along the neutrophil pathway, several distinct morphological 15 stages are seen. Myeloblast develop into promyelocytes and myelocytes, which mature and are released into the circulation as neutrophils. The one-way differentiation of cells from the CFU-GM into mature neutrophils is probably the result of acquiring specific growth/differentiation factor receptors at different stages of development.

20 Surface differentiation markers disappear or appear on the cells as they develop into granulocytes. For example, MHC class II molecules and CD38 are expressed on the CFU-GM but not on mature neutrophils. Other surface molecules acquired during the differentiation process include CD13, CD14 at low density, CD15, the  $\beta_1$  integrin, VLA-4, the  $\beta_2$  integrins CD11a, b and c associated with CD18  $\beta_2$  chains, complement receptors and 25 CD16 Fc $\gamma$  receptors.

CFU-GMs taking the monocyte pathway give rise initially to proliferating monoblasts. These differentiate into promonocytes and finally into mature circulating monocytes. Circulating monocytes are thought to be a replacement pool for tissue-resident 30 macrophages. The different forms of macrophages comprise the reticulo-endothelial system.

Like mature neutrophils, mature monocytes and macrophages lose CD34. However, unlike neutrophils, they continue to express significant levels of MHC class II molecules. These molecules are clearly important for the presentation of antigen to T cells. Monocytes also acquire many of the same surface molecules as mature neutrophils.

In addition to macrophages, most of the classical antigen-presenting cells (APCs) which include the follicular dendritic cells, Langerhans' cells and interdigitating cells are present at birth. While their origin is still unclear, it is likely that most are derived from bone-marrow stem cells. One possibility is that they are derived from the same CFU-GEMM precursor cell. Morphological, cytochemical and functional differences would then be due to local microenvironmental influences such as cytokines. Alternatively, APCs could be derived from different stem cells and represent separate lineages of differentiation.

In the first stage of differentiation into colony forming cells (such as CFU-GEMM) the HSCs express CD33 and CD34. Thus, HSCs can usually be characterised by the presence of the cell glycoprotein CD34 (and possibly CD33) at the cell surface.

In the next stage of differentiation to cells of the erythroid, myelomonocytic and megakaryotic lineages, the vital burst forming units-erythroid (BFUE) cells of the erythroid lineage carry antigens CD33 and CD34 but these antigens are lost in later differentiation. The myelomonocytic lineage which includes CFU-GM cells carry CD33 but not CD34 and this CD33 is subsequently lost. The megakaryotic lineage leads initially to CFU Mega cells which carry CD34 which is also subsequently lost.

A further significant system of antigens on HSC and other cells is the MHC (major histocompatibility complex) Class II group. It has been found that the majority of HSC carry an antigen termed DR and on differentiation express an antigen termed DP and then a further antigen termed DQ. Thus, the MHC Class II DR antigen is characteristic of relatively early stem cells.

Methods for isolation of HSCs and their maintenance and differentiation in culture are known in the art (Santiago-Schwartz *et al* 1992 J Leuk Biol 52:274-281; Charbord *et al* 1996 Br J Haematol 94: 449-454; Dao *et al* 1997 Blood 89: 446-456; Piacibello *et al* 1997 Blood 89: 2644-2653) and in WO91/09938. Methods for retroviral mediated transduction  
5 of HSCs and transfer to patients are also described (Dunbar *et al* 1996 Hum Gene Ther 7:231-253).

Engineered HSCs of the invention are administered to a patient or an at-risk individual in a suitable formulation. The formulation may include an isotonic saline solution, a buffered  
10 saline solution or a tissue-culture medium. The cells are administered by bolus injection or by infusion intravenously or directly to the site of a tumour or to the bone marrow at a concentration of for example between approximately  $10^6$  and of the order of  $10^{12}$  cells / dose.

The individual may first be treated to deplete the bone marrow of stem cells or may be  
15 treated with one or more cytokines such as G-CSF to increase the mobilisation of stem cells into the peripheral blood or one or more cytokines to enhance repopulation of bone marrow. Combinations of such treatments are also envisaged. The treatments of the invention may also be combined with currently available anti-cancer therapies.

20 In the event that the vector used for stem cell engineering encodes a pro-drug activating enzyme, the individual suffering from cancer is additionally treated with the corresponding pro-drug, administered using an appropriate regimen according to principles known in the art.

As indicated above, the present invention is based on the surprising finding that it is  
25 possible to transform one or more HSCs with *inter alia* a IRLE and for a specific purpose.

A preferred IRLE is an hypoxic response element.

The elevated expression of a therapeutic gene under hypoxic conditions can be induced by  
30 the presence of one or more hypoxic response enhancer (HRE) elements. HRE elements contain polynucleotide sequences that may be located either upstream (5') or downstream

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(3') of the promoter and/or therapeutic gene. The HRE enhancer element (HREE) is typically a *cis*-acting element, usually about 10-300 bp in length, that acts on a promoter to increase the transcription of a gene under the control of the promoter. Preferably, the promoter and enhancer elements are selected such that expression of a gene regulated by those elements is minimal in the presence of a healthy supply of oxygen and is upregulated under hypoxic or anoxic conditions.

The term "hypoxia" means a condition under which a particular organ or tissue receives an inadequate supply of oxygen.

The hypoxia response element may also be selected from, for example, the erythropoietin HRE element (HREE1), muscle pyruvate kinase (PKM), HRE element, B-enolase (enolase 3; ENO3) HRE element, endothelin-1 (ET-1)HRE element and metallothionein II (MTII) HRE element.

A further example of a hypoxia regulated enhancer is a binding element for the transcription factor HIF-1 (Dachs *et al* 1997 Nature Med 5: 515; Wang and Sememnza 1993 Proc Natl Acad Sci USA 90:4304; Firth *et al* 1994 Proc Natl Acad Sci USA 91: 6496). Hypoxia response enhancer elements have also been found in association with a number of genes including the erythropoietin (EPO) gene (Madan *et al* 1993 Proc Natl Acad Sci 90: 3928; Semenza and Wang 1992 Mol Cell Biol 1992 12: 5447-5454). Other HREs have been isolated from regulatory regions of both the muscle glycolytic enzyme pyruvate kinase (PKM) gene (Takenaka *et al* 1989 J Biol Chem 264: 2363-2367), the human muscle-specific  $\beta$ -enolase gene (ENO3; Peshavaria and Day 1991 Biochem J 275: 427-433 ) and the endothelin-1 (ET-1) gene (Inoue *et al* 1989 J Biol Chem 264: 14954-14959).

Alternatively the expression of a therapeutic gene can be regulated by glucose concentration.

For example, the glucose-regulated proteins (grp's) such as grp78 and grp94 are highly conserved proteins known to be induced by glucose deprivation (Attenello and Lee 1984 Science 226 187-190). The grp 78 gene is expressed at low levels in most normal healthy tissues under the influence of basal level promoter elements but has at least two critical "stress inducible regulatory elements" upstream of the TATA element (Attenello 1984  
5 *ibid*; Gazit *et al* 1995 Cancer Res 55: 1660-1663). Attachment to a truncated 632 base pair sequence of the 5'end of the grp78 promoter confers high inducibility to glucose deprivation on reporter genes *in vitro* (Gazit *et al* 1995 *ibid*). Furthermore, this promoter sequence in retroviral vectors was capable of driving a high level expression of a reporter  
10 gene in tumour cells in murine fibrosarcomas, particularly in central relatively ischaemic/fibrotic sites (Gazit *et al* 1995 *ibid*).

The present invention is believed to have a wide therapeutic applicability - depending on *inter alia* the selection of the one or more NOIs.

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For example, the present invention may be useful in the treatment of the disorders listed in WO-A-98/05635. For ease of reference, part of that list is now provided: cancer, inflammation or inflammatory disease, dermatological disorders, fever, cardiovascular effects, haemorrhage, coagulation and acute phase response, cachexia, anorexia, acute  
20 infection, HIV infection, shock states, graft-versus-host reactions, autoimmune disease, reperfusion injury, meningitis, migraine and aspirin-dependent anti-thrombosis; tumour growth, invasion and spread, angiogenesis, metastases, malignant, ascites and malignant pleural effusion; cerebral ischaemia, ischaemic heart disease, osteoarthritis, rheumatoid arthritis, osteoporosis, asthma, multiple sclerosis, neurodegeneration, Alzheimer's disease,  
25 atherosclerosis, stroke, vasculitis, Crohn's disease and ulcerative colitis; periodontitis, gingivitis; psoriasis, atopic dermatitis, chronic ulcers, epidermolysis bullosa; corneal ulceration, retinopathy and surgical wound healing; rhinitis, allergic conjunctivitis, eczema, anaphylaxis; restenosis, congestive heart failure, endometriosis, atherosclerosis or endosclerosis.

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In addition, or in the alternative, the present invention may be useful in the treatment of disorders listed in WO-A-98/07859. For ease of reference, part of that list is now provided: cytokine and cell proliferation/differentiation activity; immunosuppressant or immunostimulant activity (e.g. for treating immune deficiency, including infection with human immune deficiency virus; regulation of lymphocyte growth; treating cancer and many autoimmune diseases, and to prevent transplant rejection or induce tumour immunity); regulation of haematopoiesis, e.g. treatment of myeloid or lymphoid diseases; promoting growth of bone, cartilage, tendon, ligament and nerve tissue, e.g. for healing wounds, treatment of burns, ulcers and periodontal disease and neurodegeneration; inhibition or activation of follicle-stimulating hormone (modulation of fertility); chemotactic/chemokinetic activity (e.g. for mobilising specific cell types to sites of injury or infection); haemostatic and thrombolytic activity (e.g. for treating haemophilia and stroke); antiinflammatory activity (for treating e.g. septic shock or Crohn's disease); as antimicrobials; modulators of e.g. metabolism or behaviour; as analgesics; treating specific deficiency disorders; in treatment of e.g. psoriasis, in human or veterinary medicine.

In addition, or in the alternative, the present invention may be useful in the treatment of disorders listed in WO-A-98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune response, including a response not associated with inflammation; inhibit the ability of macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reaction and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases,

glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididymo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fundus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillain-Barre syndrome, Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplantation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or

cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers,  
5 natural or artificial skin tissue.

The delivery of one or more NOIs by a vector system according to the present invention may be used alone or in combination with other treatments or components of the treatment.

10 As indicated above, a preferred aspect of the present invention is based on the surprising finding that it is possible to transform one or more HSCs with a retroviral vector and for a specific purpose.

An even more preferred aspect of the present invention is based on the surprising finding  
15 that it is possible to configure lentiviral vectors in order to enhance production of the vector *in vitro* or to regulate gene expression *in vivo* in response to a normal physiological signal.

Previous work has shown that it is possible to develop vectors based on members of the  
20 lentivirus family which includes HIV-1, HIV-2, SIV, FIV and ELAV. To date all of the vectors express the therapeutic genes either constitutively or in response to the natural regulatory signals of the viral vectors themselves. Neither configuration has broad utility for the treatment of disease where controlled levels of gene expression are required. We now describe, for the first time, lentiviral vectors that are responsive to hypoxia and to  
25 agents that mimic hypoxia. This regulation can be harnessed *in vitro* to enhance production of the vector and it can be used *in vivo* to regulate gene expression in response to a normal physiological signal. Such vectors have utility in a wide range of diseases where ischaemia is a feature, for example, cardiovascular disease, peripheral arterial disease, cancer and arthritis.

Despite intense research there has been no description to date of a lentiviral vector that contains a regulated gene and it is not clear from the literature that such a vector could be produced. Such a vector would have broad utility for a range of diseases. We have now produced a set of lentiviral vectors that are regulated by tissue physiology and by a chemical modulator. These vectors can be configured where the expression cassette is place internally to the vector as described above. However we have shown that there is further advantage to configuring such vectors as single transcription unit vectors. In this configuration the resultant duplication of the regulatory sequence enhances the response. The regulatory system that we have studied exploits the fact that gene expression is activated in response to ischaemia. The physiological markers of ischaemia are low oxygen, low pH and low glucose and these conditions are sensed in cells to result in activation of a restricted set of genes. One such set of genes contain sequences in the DNA that mediate a response principally to hypoxia these are the hypoxia response elements (HRE). The use of these elements to drive the expression of genes in plasmid, retroviral and adenoviral vectors has been described previously in patent applications [PCT/GB95/00322; PCT/GB97/02709] and in the literature (e.g. Dachs et al 1997 *ibid*). It has not however been demonstrated previously that they have utility in lentiviral vectors.

In addition to responding to hypoxia the HRE elements are known to respond to chemical inducers that mimic hypoxia. Two of these are known, these are cobalt and desferrioxamine (Meliillo et al 1996 J. Biol. Chem 272, 12236-12243; Wang and Semenza 1993, Blood, 82, 3610).

The invention is applicable to any lentiviral vector for use in any cell type for use in any disease where ischaemia is evident or for use in any disease where the chemical activator desferrioxamine or analogous chemicals might be used, for example neuroblastoma (Blatt 1994, Anticancer Res., 14, 2109), beta thalassemia (Giardina and Grady 1995, Semin. Hematol. 32, 304, Alzheimers disease (Crapper et al 1991, The Lancet, 337, 1304, VEGF deficiency (Beerrepoot et al 1996, 56, 3747), Eprythropoetin deficiency (Wang and Semenza op cit) and enhancement of tumour chemotherapy (Voest et al 1993, Cancer Chemother. Pharmacol. 31, 357).

As it is well known in the art, a vector is a tool that allows or facilitates the transfer of an entity from one environment to another. In accordance with the present invention, and by way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

The vector can be delivered by viral or non-viral techniques.

Non-viral delivery systems include but are not limited to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a gene to a target mammalian cell.

Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), multivalent cations such as spermine, cationic lipids or polylysine, 1, 2,-bis (oleoyloxy)-3-(trimethylammonio) propane (DOTAP)-cholesterol complexes (Wolff and Trubetskoy 1998 Nature Biotechnology 16: 421) and combinations thereof.

Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, retroviral vector, lentiviral vector, baculoviral vector. Other examples of vectors include *ex vivo* delivery systems, which include but are not limited to DNA transfection methods such as electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection.

The vector may be a plasmid DNA vector. Suitable recombinant viral vectors include adenovirus vectors, adeno-associated viral (AAV) vectors, Herpes-virus vectors, or retroviral vectors which are preferred. In the case of viral vectors, gene delivery is mediated by viral infection of a target cell.

5

The vector of the present invention may be configured as a split-intron vector. A split intron vector is described in GB 9720465.5 and now in a PCT patent application claiming priority therefrom (titled VECTOR) and filed on the same date as the filing of this PCT patent application. For ease of reference, that PCT patent application claims: A retroviral  
10 vector comprising a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector; wherein the retroviral pro-vector comprises a first  
15 nucleotide sequence (NS) capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS; such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector. In this application, the first NOI is an NOI as herein defined. Teachings on the split intron aspect are provided at the end of  
20 the Example Section provided below. These teachings provide information on this preferred aspect - namely how to construct a split intron viral vector.

Thus, in this preferred aspect of the present invention there is provided a modified cell comprising an element that is active in that cell; and an NOI; wherein the modified cell is  
25 prepared by transforming a cell by viral transduction with one or more retroviral vectors wherein at least one of which comprises the NOI; wherein the retroviral vector comprises a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional  
30 splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector; wherein the retroviral pro-vector comprises a first nucleotide sequence (NS) capable of

yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS; such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.

5

The vector of the present invention may be an adenoviral vector.

The adenovirus is a double-stranded, linear DNA virus that does not go through an RNA intermediate. There are over 50 different human serotypes of adenovirus divided into 6 subgroups based on the genetic sequence homology. The natural target of adenovirus is the respiratory and gastrointestinal epithelia, generally giving rise to only mild symptoms. Serotypes 2 and 5 (with 95% sequence homology) are most commonly used in adenoviral vector systems and are normally associated with upper respiratory tract infections in the young.

15

Adenoviruses are nonenveloped, regular icosahedrons. A typical adenovirus comprises a 140nm encapsidated DNA virus. The icosahedral symmetry of the virus is composed of 152 capsomeres: 240 hexons and 12 pentons. The core of the particle contains the 36 kb linear duplex DNA which is covalently associated at the 5' ends with the Terminal Protein (TP) which acts as a primer for DNA replication. The DNA has inverted terminal repeats (ITR) and the length of these varies with the serotype.

20

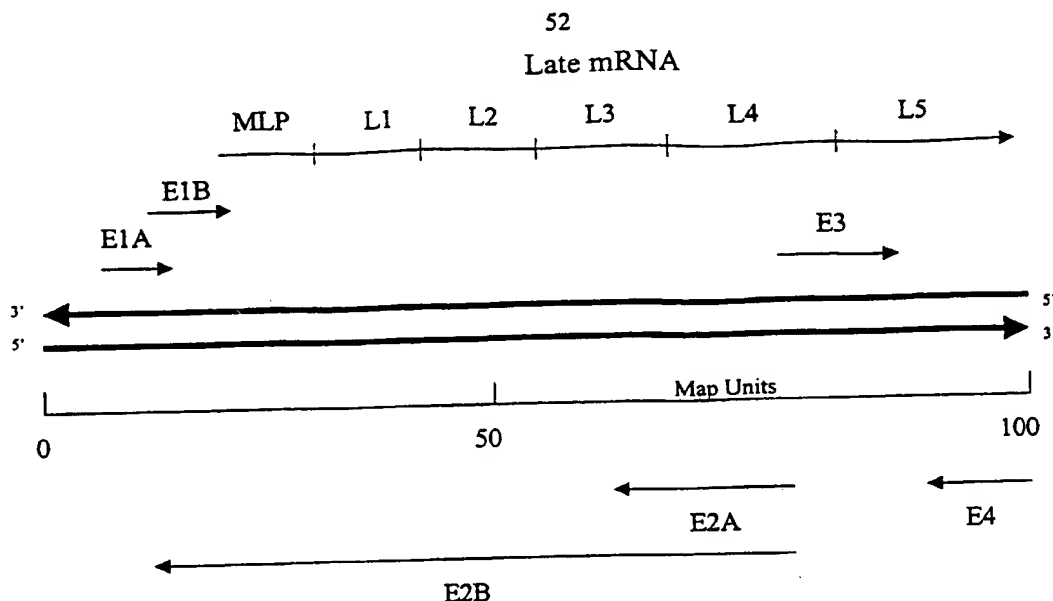
Entry of adenovirus into cells involves a series of distinct events. Attachment of the virus to the cell occurs via an interaction between the viral fibre (37nm) and the fibre receptors on the cell. This receptor has recently been identified for Ad2/5 serotypes and designated as CAR (Coxsackie and Adeno Receptor, Tomko *et al* (1997 Proc Natl Acad Sci 94: 3352-3358). Internalisation of the virus into the endosome via the cellular  $\alpha v \beta 3$  and  $\alpha v \beta 5$  integrins is mediated by and viral RGD sequence in the penton-base capsid protein (Wickham *et al.*, 1993 Cell 73: 309-319). Following internalisation, the endosome is disrupted by a process known as endosomolysis, an event which is believed to be preferentially promoted by the cellular  $\alpha v \beta 5$  integrin (Wickham *et al.*, 1994 J Cell Biol

30

127: 257-264). In addition, there is recent evidence that the Ad5 fibre knob binds with high affinity to the MHC class 1  $\alpha 2$  domain at the surface of certain cell types including human epithelial and B lymphoblast cells (Hong *et al.*, 1997 EMBO 16: 2294-2306).

- 5 Subsequently the virus is translocated to the nucleus where activation of the early regions occurs and is shortly followed by DNA replication and activation of the late regions. Transcription, replication and packaging of the adenoviral DNA requires both host and viral functional protein machinery.
- 10 Viral gene expression can be divided into early (E) and late (L) phases. The late phase is defined by the onset of viral DNA replication. Adenovirus structural proteins are generally synthesised during the late phase. Following adenovirus infection, host cellular mRNA and protein synthesis is inhibited in cells infected with most serotypes. The adenovirus lytic cycle with adenovirus 2 and adenovirus 5 is very efficient and results in
- 15 approximately 10, 000 virions per infected cell along with the synthesis of excess viral protein and DNA that is not incorporated into the virion. Early adenovirus transcription is a complicated sequence of interrelated biochemical events but it entails essentially the synthesis of viral RNAs prior to the onset of DNA replication.
- 20 The schematic diagram below is of the adenovirus genome showing the relative direction and position of early and late gene transcription:





The organisation of the adenovirus genome is similar in all of the adenovirus groups and specific functions are generally positioned at identical locations for each serotype studied. Early cytoplasmic messenger RNAs are complementary to four defined, noncontiguous regions on the viral DNA. These regions are designated E1-E4. The early transcripts have  
 5 been classified into an array of intermediate early (E1a), delayed early (E1b, E2a, E2b, E3 and E4), and intermediate regions.

The early genes are expressed about 6-8 hours after infection and are driven from 7 promoters in gene blocks E1-4:

10

The E1a region is involved in transcriptional transactivation of viral and cellular genes as well as transcriptional repression of other sequences. The E1a gene exerts an important control function on all of the other early adenovirus messenger RNAs. In normal tissues, in order to transcribe regions E1b, E2a, E2b, E3 or E4 efficiently, active E1a product is  
 15 required. However, the E1a function may be bypassed. Cells may be manipulated to provide E1a-like functions or may naturally contain such functions. The virus may also be manipulated to bypass the E1a function. The viral packaging signal overlaps with the E1a enhancer (194-358 nt).

20 The E1b region influences viral and cellular metabolism and host protein shut-off. It also

includes the gene encoding the pIX protein (3525-4088 nt) which is required for packaging of the full length viral DNA and is important for the thermostability of the virus. The E1b region is required for the normal progression of viral events late in infection. The E1b product acts in the host nucleus. Mutants generated within the E1b sequences exhibit  
5 diminished late viral mRNA accumulation as well as impairment in the inhibition of host cellular transport normally observed late in adenovirus infection. E1b is required for altering functions of the host cell such that processing and transport are shifted in favour of viral late gene products. These products then result in viral packaging and release of virions. E1b produces a 19 kD protein that prevents apoptosis. E1b also produces a 55 kD  
10 protein that binds to p53. For a review on adenoviruses and their replication, see WO 96/17053.

The E2 region is essential as it encodes the 72 kDa DNA binding protein, DNA polymerase and the 80 kDa precursor of the 55 kDa Terminal Protein (TP) needed for protein priming  
15 to initiate DNA synthesis.

A 19 kDa protein (gp19K) is encoded within the E3 region and has been implicated in modulating the host immune response to the virus. Expression of this protein is upregulated in response to TNF alpha during the first phase of the infection and this then  
20 binds and prevents migration of the MHC class I antigens to the epithelial surface, thereby dampening the recognition of the adenoviral infected cells by the cytotoxic T lymphocytes. The E3 region is dispensable in *in vitro* studies and can be removed by deletion of a 1.9 kb *XbaI* fragment.

25 The E4 region is concerned with decreasing the host protein synthesis and increasing the DNA replication of the virus.

There are 5 families of late genes and all are initiated from the major late promoter. The expression of the late genes includes a very complex post-transcriptional control  
30 mechanism involving RNA splicing. The fibre protein is encoded within the L5 region. The adenoviral genome is flanked by the inverted terminal repeat which in Ad5 is 103 bp

and is essential for DNA replication. 30-40 hours post infection viral production is complete.

Adenoviruses may be converted for use as vectors for gene transfer by deleting the E1 gene, which is important for the induction of the E2, E3 and E4 promoters. The E1-replication defective virus may be propagated in a cell line that provides the E1 polypeptides *in trans*, such as the human embryonic kidney cell line 293. A therapeutic gene or genes can be inserted by recombination in place of the E1 gene. Expression of the gene is driven from either the E1 promoter or a heterologous promoter.

Even more attenuated adenoviral vectors have been developed by deleting some or all of the E4 open reading frames (ORFs). However, certain second generation vectors appear not to give longer-term gene expression, even though the DNA seems to be maintained. Thus, it appears that the function of one or more of the E4 ORFs may be to enhance gene expression from at least certain viral promoters carried by the virus.

An alternative approach to making a more defective virus has been to "gut" the virus completely maintaining only the terminal repeats required for viral replication. The "guttled" or "gutless" viruses can be grown to high titres with a first generation helper virus in the 293 cell line but it has been difficult to separate the "guttled" vector from the helper virus.

Replication-competent adenoviruses can also be used for gene therapy. For example, the E1a gene can be inserted into a first generation virus under the regulation of a tumour-specific promoter. In theory, following injection of the virus into a tumour, it could replicate specifically in the tumour but not in the surrounding normal cells. This type of vector could be used either to kill tumour cells directly by lysis or to deliver a "suicide gene" such as the herpes-simplex-virus thymidine-kinase gene (HSV *tk*) which can kill infected and bystander cells following treatment with ganciclovir. Alternatively, an adenovirus defective only for E1b has been used specifically for antitumour treatment in phase-1 clinical trials. The polypeptides encoded by E1b are able to block p53-mediated

apoptosis, preventing the cell from killing itself in response to viral infection. Thus, in normal nontumour cells, in the absence of E1b, the virus is unable to block apoptosis and is thus unable to produce infectious virus and spread. In tumour cells deficient in p53, the E1b defective virus can grow and spread to adjacent p53-defective tumour cells but not to normal cells. Again, this type of vector could also be used to deliver a therapeutic gene such as HSV *tk*.

The adenovirus provides advantages as a vector for gene delivery over other gene therapy vector systems for the following reasons:

10

It is a double stranded DNA nonenveloped virus that is capable of *in vivo* and *in vitro* transduction of a broad range of cell types of human and non-human origin. These cells include respiratory airway epithelial cells, hepatocytes, muscle cells, cardiac myocytes, synoviocytes, primary mammary epithelial cells and post-mitotically terminally differentiated non-replicating cells such as macrophages neurons (with perhaps the important exception of some lymphoid cells including monocytes).

15

Adenoviral vectors are capable of transducing both dividing and non dividing cells. This is very important for diseases, such as cystic fibrosis, in which the affected cells in the lung epithelium, have a slow turnover rate. In fact, several trials are underway utilising adenovirus-mediated transfer of cystic fibrosis transporter (CFTR) into the lungs of afflicted adult cystic fibrosis patients.

20

Adenoviruses have been used as vectors for gene therapy and for expression of heterologous genes. First generation recombinant adenovirus vectors (E1/E3 deleted) can accommodate 7-8kb of foreign insert DNA, second (E1/E3/E4 deleted) can third ("gutless") generation can carry much larger DNA inserts. Recombinant adenovirus vectors can be propagated and stocked to very high titres. Adenoviral vector replication in complementing cell lines may produce very high titres of up to  $10^{13}$  viral particles per ml. Adenovirus is thus one of the best systems to study the expression of genes in primary non-replicative cells.

30

The expression of viral or foreign genes from the adenovirus genome does not require a replicating cell. Adenoviral vectors enter cells by receptor mediated endocytosis. Once inside the cell, adenovirus vectors rarely integrate into the host chromosome. Instead, it functions episomally (independently from the host genome) as a linear genome in the host nucleus. Hence the use of recombinant adenovirus alleviates the problems and avoids the risks associated with random integration into the host genome.

There is no association of human malignancy with adenovirus infection. Attenuated adenoviral strains have been developed and have been used in humans as live vaccines.

10

However, current adenoviral vectors suffer from some major limitations for *in vivo* therapeutic use. These include: (i) transient gene expression- the adenoviral vector generally remains episomal and does not replicate so that it is not passed onto subsequent progeny (ii) because of its inability to replicate, target cell proliferation can lead to dilution of the vector (iii) an immunological response raised against the adenoviral proteins so that cells expressing adenoviral proteins, even at a low level, are destroyed (iv) an inability to achieve an effective therapeutic index since *in vivo* delivery leads to an uptake of the vector and expression of the delivered genes in only a proportion of target cells (v) the broad target range of adenoviruses can be problematic with gene therapy approaches that need to be directed to diseased tissue with minimal toxicity of normal tissues. Any additional controls that can be used to focus therapy in the required compartment would be advantageous with this vector system.

Adenoviral vectors for use in the invention may be derived from a human adenovirus or an adenovirus which does not normally infect humans. Preferably the vectors are derived from adenovirus type 2 or adenovirus type 5 (Ad2 or Ad5) or a mouse adenovirus or an avian adenovirus such as CELO virus (Cotton *et al* 1993 J Virol 67:3777-3785). The vectors may be replication competent adenoviral vectors but are more preferably defective adenoviral vectors. Adenoviral vectors may be rendered defective by deletion of one or more components necessary for replication of the virus. Typically, each adenoviral vector contains at least a deletion in the E1 region. For production of infectious adenoviral vector

particles, this deletion may be complemented by passage of the virus in a human embryo fibroblast cell line such as human 293 cell line, containing an integrated copy of the left portion of Ad5, including the E1 gene. The capacity for insertion of heterologous DNA into such vectors can be up to approximately 7 kb. Thus such vectors are useful for construction of a system according to the invention comprising three separate recombinant vectors each containing one of the essential transcription units for construction of the retroviral secondary vector.

To date, there has been no published data on physiologically regulated recombinant adenoviruses. However, a number of tissue specific promoters which have been explored in the context of adenoviral vectors, five of which are described below:

The Pancreatitis-associated Protein I promoter (which is strongly induced during the acute phase of pancreatitis) shows a 90-fold induction of CAT activity in AR-42J pancreatic cell line after stimulation with a combination of IL6 and dexamethasone *in vitro* (Duseti *et al* 1997 J. Biol. Chem. 272(9) 5800-5804). Mouse *in vivo* experiments (adenovirus administered via intravenous injection into the tail vein) showed a 10 -fold higher activity in animals with pancreatitis (low activity of CAT was observed in some other tissues of the control animal).

A recombinant adenovirus containing the murine alpha-fetoprotein promoter was constructed to direct hepatocellular (HCC) specific expression of the human interleukin-2 gene. IL-2 expression was 3-4 fold higher in AFP-producing HCC cell lines compared to non-AFP producing non-HCC lines (Bui *et al* 1997 Human Gene Therapy. 8 2173-2182). Intratumoral injection of the AdVAFP1-IL2 into Hep3B tumours established in the dorsal flanks of CB-17/SCID mice resulted in substantial growth arrest and tumour regression after 3 injections.

A recombinant adenovirus was constructed containing a segment the cardiac troponin T (cTnT) promoter controlling the expression of either lacZ, EGFP or the sarcoendoplasmic reticulum Ca-ATPase (SERCA) gene (Inesi *et al* 1998 American J. Physiol. 274(3,1)

C645-C653). The recombinant viruses were then used to transduce chick myocytes and fibroblasts in culture to show that expression from the cTnT promoter was restricted to cardiac myocytes.

5 The smooth muscle specific promoter (SM22 $\alpha$ ) was used to drive expression of the bacteriophage lacZ reporter gene in a recombinant adenovirus (Kim *et al* (1997 J. Clin. Invest. 100(5) 1006-1014). Expression was detected in primary rat aortic SMCs and immortalised A7r5 SMCs, but not in HUVECs or NIH3T3 cells. The recombinant adenovirus was injected intravenously into Sprague Dawley rats and although expression from the  
10 CMVlacZ adenovirus was detected throughout the liver and lung no expression in either of these tissues was detected from the SM22 driven lacZ adenovirus, restricting transgene expression to SMCs. The conclusion of the paper states that the AdSMCC-lacZ expression was restricted to visceral and vascular SMCs when the virus was administered intraarterially, intravenously or intramuscularly.

15 The KDR and E-selectin promoters were engineered to upregulate the expression of murine TNF- $\alpha$  from a SIN retroviral vector in endothelial cells (Jaggar *et al* 1997 Human Gene Therapy 8(18) 2239-2247). A 10 fold increase in expression from these promoter elements within sEND endothelial cells as compared to NIH-353 fibroblasts was  
20 observed.

If the features of adenoviruses are combined with the genetic stability of retro/lentiviruses then essentially the adenovirus can be used to transduce target cells to become transient retroviral producer cells that could stably infect neighbouring cells.

25 Preferred vectors for use in accordance with the present invention are recombinant viral vectors, in particular recombinant retroviral vectors.

Preferred vectors for use in accordance with the present invention are recombinant viral  
30 vectors, in particular recombinant adenoviral vectors.

Preferred vectors for use in accordance with the present invention are recombinant viral vectors, in particular a combination of adenoviral and retroviral vectors.

5 The term "recombinant retroviral vector" (RRV) refers to a vector with sufficient retroviral genetic information to allow packaging of an RNA genome, in the presence of packaging components, into a viral particle capable of infecting a target cell. Infection of the target cell includes reverse transcription and integration into the target cell genome. The RRV in use carries non-viral coding sequences which are to be delivered by the vector to the target cell. An RRV is incapable of independent replication to produce infectious retroviral  
10 particles within the final target cell. Usually the RRV lacks a functional *gag-pol* and/or *env* gene and/or other genes essential for replication.

Where the invention uses a vector for delivery of an NOI or genes to HSCs *in vivo*, the vector is preferably a targeted vector capable of targeting CD34<sup>+</sup> HSCs.

15

The term "targeted vector" refers to a vector whose ability to infect/transfect a cell or to be expressed in the target cell is restricted to certain cell types within the host organism, usually cells having a common or similar phenotype.

20 An example of a targeted vector is a targeted retroviral vector with a genetically modified envelope protein which binds to cell surface molecules found only on a limited number of cell types in the host organism. Another example of a targeted vector is one which contains promoter and/or enhancer elements which only permit expression of one or more retroviral transcripts in a proportion of the cell types of the host organism. Thus, the vector may be  
25 provided with a ligand specific for CD34, such as an antibody or an immunoglobulin-like molecule directed against CD34. On introduction into an individual to be treated such a vector will specifically transfect CD34<sup>+</sup> HSCs. The vector may be administered systemically, to the peripheral circulation.

30 The retroviral vector particle according to the invention will also be capable of transducing cells which are slowly-dividing, and which non-lentiviruses such as MLV would not be



able to efficiently transduce. Slowly-dividing cells divide once in about every three to four days including certain tumour cells. Although tumours contain rapidly dividing cells, some tumour cells especially those in the centre of the tumour, divide infrequently.

- 5 Examples of tumours that may be treated by the present invention include but are not limited to: sarcomas including osteogenic and soft tissue sarcomas, carcinomas such as breast, lung, bladder, thyroid, prostate, colon, rectum, pancreas, stomach, liver, uterine, and ovarian carcinoma, lymphomas including Hodgkin and non-Hodgkin lymphomas, neuroblastoma, melanoma, myeloma, Wilms tumour, and leukemias, including acute  
10 lymphoblastic leukemia and acute myeloblastic leukemia, gliomas and retinoblastomas.

Alternatively the target cell may be a growth-arrested cell capable of undergoing cell division such as a cell in a central portion of a tumour mass or a stem cell such as a HSC or a CD34<sup>+</sup> cell. As a further alternative, the target cell may be a precursor of a differentiated  
15 cell such as a monocyte precursor, a CD33<sup>+</sup> cell, or a myeloid precursor. As a further alternative, the target cell may be a differentiated cell such as a neuron, astrocyte, glial cell, microglial cell, macrophage, monocyte, epithelial cell, endothelial cell or hepatocyte. Target cells may be transduced either *in vitro* after isolation from a human individual or may be transduced directly *in vivo*.

20

Additional vector components which are standard in the art will be provided for other aspects of vector function such as vector maintenance, nuclear localisation, replication, and integration as appropriate.

- 25 Where the HSCs are removed from the individual to be treated and are transfected or transduced with the vector *in vitro*, the cells are generally expanded in culture prior to and after introduction of the NOI or NOIs. When cultured *in vitro* under appropriate conditions or when appropriate signals are received *in vivo*, HSC have the capacity to differentiate into, among other cell types, endothelial cells, myeloid cells, dendritic cells and immune  
30 effector cells such as neutrophils, lymphocytes, mononuclear phagocytes and NK cells.

This involves the use of tissue culture methods which are known in the art and include exposure to cytokines and/or growth factors for the maintenance of HSCs (Santiago-Schwartz *et al* 1992 J Leuk Biol 52: 274-281; Charbord *et al* 1996 Br J Haematol 94: 449-454; Dao *et al* 1997 Blood 89: 446-454; Piacibello *et al* 1997 Blood 89: 2644-2653).

- 5 Agents which induce the differentiation of the HSCs may also be added.

As indicated, the vector of the present invention may be a delivered to a target site by a viral or a non-viral vector.

- 10 The invention also provides reagents and methods for use in treating diseases such as cancer, and also reagents and methods for use in preventative medicine. Thus the NOIs used in the invention may have a therapeutic effect via prophylaxis. For example, where an increased risk of developing cancer is diagnosed, the invention may be used to vaccinate the at-risk individual.

15

The present invention also provides a pharmaceutical composition for treating an individual by gene therapy, wherein the composition comprises a therapeutically effective amount of the retroviral vector of the present invention comprising one or more deliverable therapeutic and/or diagnostic NOI(s) or a viral particle produced by or obtained from same.

- 20 The pharmaceutical composition may be for human or animal usage. Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient.

- The composition may optionally comprise a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).
- 25
- 30

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules  
5 either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or  
10 monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

Suitability for prophylaxis may be based on genetic predisposition to cancer, for example  
15 cancer of the breast or ovary because of one or more mutations in a BRCA-1 gene, a BRCA-2 gene (Cornelisse *et al* 1996 Pathol Res Pract 192: 684-693) or another relevant gene.

In accordance with the invention, standard molecular biology techniques may be used  
20 which are within the level of skill in the art. Such techniques are fully described in the literature. See for example; Sambrook *et al* (1989) Molecular Cloning; a laboratory manual; Hames and Glover (1985 - 1997) DNA Cloning: a practical approach, Volumes I-IV (second edition); Methods for the engineering of immunoglobulin genes are given in McCafferty *et al* (1996) "Antibody Engineering: A Practical Approach".

25

In summation, the present invention relates to: a delivery system suitable for introducing one or more NOIs into a HSC.

The present invention also relates to a MHSC containing a vector comprising one or more  
30 NOIs.

The present invention also relates to a vaccine comprising the aforementioned vector and/or MHSC.

5 A preferred aspect of the present invention relates to uses of any of the aforementioned products in the treatment or prevention of a condition characterised by ischaemia, hypoxia or low glucose; particularly, but not exclusively, a condition such as cancer, cerebral malaria, ischaemic heart disease or rheumatoid arthritis.

10 In a further broad aspect, the present invention provides a modified HSC (MHSC) which comprises a responsive element that comprises an element that is operable in a macrophage ("macrophage responsive element").

15 Thus, the present invention also provides a modified differentiated cell (preferably terminally differentiated cell) comprising at least one expressable nucleotide sequence of interest (NOI) wherein the or each NOI is operably linked to one or more responsive element(s) active in that differentiated cell.

20 Thus, in a preferred aspect, the present invention also provides a modified macrophage comprising at least one expressable nucleotide sequence of interest (NOI) wherein the or each NOI is operably linked to one or more responsive element(s) active in that differentiated cell.

25 Thus, in a preferred aspect, the present invention also provides a modified endothelial cell comprising at least one expressable nucleotide sequence of interest (NOI) wherein the or each NOI is operably linked to one or more responsive element(s) active in that differentiated cell.

With these broad aspects of the present invention, preferably the HRE of the present invention is a highly preferred component of such a responsive element.

Preferably, the differentiated cell is derived from the MHSC. This aspect is advantageous as it provides a means for providing for selective expression in or by or from, for example, a macrophage that has been differentiated from the MHSC.

- 5 In a further broad aspect, the present invention provides a modified cell, which cell may be a differentiated or undifferentiated cell which undifferentiated cell is capable of being differentiated to a differentiated cell, which modified cell comprises an element that is active in that cell (preferably only active in that cell type); and an NOI (as defined above); wherein the modified cell is prepared by transforming a cell by viral transduction,  
10 preferably by adenoviral transduction and/or lentiviral transduction. Here, the element is preferably the ILRE of the present invention.

In a further broad aspect, the present invention provides a hybrid viral vector system for *in vivo* gene delivery, which system comprises one or more primary viral vectors which  
15 encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell.

Preferably the primary vector is obtainable from or is based on a adenoviral vector  
20 and/or the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector.

The invention will now be further described by way of examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in  
25 any way to limit the scope of the invention.

Reference is made to the following Figures:

Figure 1 which shows nucleotide sequences that are responsive to hypoxia;

Figure 2 which is a pictorial representation of Plasmid OB37. This plasmid contains the OBHRE promoter;

Figure 3 which shows the induction of B-galactosidase expression in breast cancer cells  
5 transduced with Xiavector;

Figure 4a which shows a synthetic hypoxia responsive promoter in combination with the SV40 minimal promoter;

10 Figure 4b which illustrates the relative strengths of synthetic hypoxic responsive promoters. The bars indicate the level of reporter gene activity. The numbers above the bars give the fold induction;

Figure 5 presents Western blots. Protein extracts were prepared from primary human  
15 macrophages and human breast cancer T47D cells and analysed by Western blotting. The antibodies were monoclonal antibodies raised against purified HIF-1 and EPAS proteins. The top panel shows the filter probed with EPAS antibody and the bottom panel shows the filter probed with the HIF-1 antibody;

20 Figure 6 which shows that OBHRE1 mediates hypoxic induction in macrophages;

Figure 7 which shows the nucleotide sequence of XiaMac and synthetic hypoxia responsive macrophage specific promoter;

25 Figure 8 which shows that hypoxia activation of a reporter gene driven by the XiaMac promoter (labelled OBHREMAC) relative to the CMV promoter in macrophages and breast cancer cell lines;

Figure 9 which shows that the HRE is critical for the hypoxic response of the XiaMac  
30 promoter. The HRE was deleted in XiaMac (XiaMac-HRE) and no induction by hypoxia is observed;

contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue.

During the process of infection, a retrovirus initially attaches to a specific cell surface receptor. On entry into the susceptible host cell, the retroviral RNA genome is then copied to DNA by the virally encoded reverse transcriptase which is carried inside the parent virus. This DNA is transported to the host cell nucleus where it subsequently integrates into the host genome. At this stage, it is typically referred to as the provirus. The provirus is stable in the host chromosome during cell division and is transcribed like other cellular proteins. The provirus encodes the proteins and packaging machinery required to make more virus, which can leave the cell by a process sometimes called "budding".

As already indicated, each retroviral genome comprises genes called *gag*, *pol* and *env* which code for virion proteins and enzymes. In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. They also serve as enhancer-promoter sequences. In other words, the LTRs can control the expression of the viral gene. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence located at the 5' end of the viral genome.

The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

For ease of understanding, simple, generic structures (not to scale) of the RNA and the DNA forms of the retroviral genome are presented in Figure 33 in which the elementary features of the LTRs and the relative positioning of *gag*, *pol* and *env* are indicated.

Figure 10 which provides an outline of a strategy to regulate our hypoxia response promoter via an autoregulatory circuit involving interferon gamma and an IRE;

- 5 Figure 11 shows the nucleotide sequence of the XiaMacIRE sequence that is only active in the presence of hypoxia and interferon gamma;

Figure 12 is a schematic diagram of a hypoxia regulated lentiviral vector targeted to vascular endothelium by the e-selectin or KDR promoter;

10

Figure 13 which shows the sequences of the WTPGK and MUTPGK;

Figure 14 which shows a pictorial representation of a pKAHRE construct;

- 15 Figure 15a shows a schematic map of a retroviral XiaGen-P450 vector comprising a therapeutic gene under the control of an HRE;

Figure 15b shows an analysis of the induction of XiaGen-P450 (a Xiavector retrovirus) by hypoxia. Cells stain dark when there is induction;

20

Figure 16a shows a pictorial representation of a plasmid map of pEGASUS;

Figure 16b shows a pictorial representation of a plasmid map of pONY2.1;

- 25 Figure 16c shows a pictorial representation of a plasmid map of pONYHRELucLac;  
Figure 16d shows a pictorial representation of a plasmid map of pEGHRELacZ;

Figure 17 is schematics representation of pSecTSP-1 and pEGHRE-TSP1;

- 30 Figure 18 shows a pictorial representation of a Pegasus vector expressing LacZ was plated onto cells in culture. Cells were then placed in normoxia or hypoxia. Under hypoxia the



reporter gene is expressed and hence B-galactosidase enzyme is expressed allowing the cells to be counted. This gives the titre of the vector. This is 2 logs higher under hypoxia indicating that the reporter gene is preferentially active under this condition;

- 5 Figure 19 shows hypoxia mediated activation of a luciferase reporter in a lentiviral vector;

Figure 20a shows a hypoxia responsive EIAV vector configured as a single transcription unit;

- 10 Figure 20b shows a hypoxia responsive autoregulated EIAV vector configured as a single transcription unit;

Figure 21 shows a pictorial representation of pE1sp1A and pJM17;

- 15 Figure 22 shows a scheme for constructing recombinant adenoviral vectors. Adeno PGKLacZ is the OBHRElacZ cassette from OB37 inserted into the Microbix transfer vector pE1sp1A;

- 20 Figure 23 shows hypoxic induction (0.1%) from PGKLacZ Ad transduced Chiang Liver cells;

Figure 24 shows hypoxic regulation of  $\beta$ -galactosidase gene expression in primary human macrophages transduced with AdHRE LacZ;

- 25 Figure 25a shows a pictorial representation of a plasmid map of pE1sp1A;

Figure 25b shows a pictorial representation of a plasmid map of pE1HREPG;

Figure 25c shows a pictorial representation of a plasmid map of pE1CMVPG;

- 30 Figure 26 shows a pictorial representation of a plasmid map of pE1RevE;

Figure 27 shows a pictorial representation of a plasmid map of pE1HORSE3.1;

Figure 28 shows a pictorial representation of a plasmid map of pE1PEGASUS4;

5 Figure 29 shows a pictorial representation of a plasmid map of pCI-Neo;

Figure 30 shows a pictorial representation of a plasmid map of pCI-Rab;

Figure 31 shows a pictorial representation of a Plasmid map of pE1Rab;

10

Figure 32 shows a hypoxia responsive ELAV vector containing two therapeutic genes; and

Figure 33 is a schematic diagram.

## 15 **EXAMPLES**

### **Example 1: Construction of ischaemia responsive promoters**

The ischaemia response element (ILRE) that can be used in this invention can be taken  
20 from any gene that is responsive to ischaemia. Hypoxia is one of the conditions that are  
associated with ischaemia and hypoxia responsive promoters are particularly useful. Some  
examples of suitable sequences are shown in Figure 1. Suitable sequences will often be  
found in DNA that is upstream (5') to the coding sequence but they are not necessarily  
present in such locations. Sequences within introns and downstream of the coding  
25 sequence can also mediate a response to hypoxia. For example sequences in the  
5'untranslated region of the Epo gene are well known to mediate a transcriptional response  
to hypoxia and sequences in the 3' region of the VEGF gene mediate a translational  
response to hypoxia (Bunn and Poyton 1996, *Physiol Rev* 76, 839).

30 The ILRE can be combined with the promoter elements that are resident in the LTR of  
retroviral vectors or in other combinations with constitutive and tissue specific promoters.

69  
In particular a combination of the PGK HRE with the elements from the SV40 promoter is useful as described below.

### OBHRE1: A promoter based on the murine PGK HRE

5 Synthetic oligonucleotides were synthesised encompassing hypoxia response element (HRE) sequences and cloned as BglII/BamHI fragments into the BamHI site of the pGL3 promoter plasmid (Promega accession no U47298). PGK sequences were synthesised as XbaI/NheI and cloned into the Nhe I site of this vector. pGL3 is an enhancerless  
10 expression plasmid with a minimal SV40 promoter upstream of a luciferase coding sequence. Insertion of the HRE at this site places it upstream of the minimal SV40 promoter. Luciferase assays were performed to compare function of this element in normoxia and hypoxia (0.1% oxygen) and to relate promoter strength to that of SV40 and CMV. The trimer encompassing -307/-290 sequence of murine PGK in the natural  
15 orientation (Firth et al 1995, J Biol Chem 270, 21021) linked to the SV40 promoter is shown.

GCTAGAGTCGTGCAGGACGTGACATCTAGTGTCGTGCAGGACGTGACA  
 HRE HRE  
 20 TCTAGTGTCGTGCAGGACGTGACAGCTAGCCCGGGCTCGAGATCTGCG  
 HRE  
 ATCTGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCC  
 SP 1 SP 1  
CATCCCGCCCCTAACTCCGCCCAGTTCGGCCCATCTCCGCCCCATCG  
 25 (SP 1) SP 1 SP 1 SP 1  
 CTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTG  
 start start

The promoter sequence defined as OBHRE is present in the plasmid OB37 that is  
30 described in Figure 2.

OBHRE1 is a novel promoter.

The HRE also functions in combination with the promoter elements in retroviral LTRs for example as shown below the MLV LTR.

5

**PGK derived enhancer sequences in the context of the MLV retroviral promoter**

PGK trimer in context of MLV retroviral promoter, forward (natural) orientation. This is identical to OB HRE with the sequences placed upstream of the Moloney MLV retroviral promoter instead of SV40. Sequence shown up to transcription start.

10

15

AGCTAGCCTAGCGTCGTGCAGGACGTGACATCTAGTGTCGTGCAGGACGTGAC  
ATCTAGTGTCGTGCAGGACGTGACATCTAGAGAACCATCAGATGTTTCCAGGG  
TGCCCCAAGGACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATCAGTTC  
GCTTCTCGCTTCTGTTCGCGCGCTTCTGCTCCCCGAGCTCAATAAAAGAGCCCA  
CAACCCCTCACTCGG

20

PGK trimer in context of MLV retroviral promoter, reverse orientation. Sequence shown up to transcription start

25

AAGCTAGCTGTACGTCCTGCACGACACTAGATGTACGTCCTGCACGACACT  
AGATGTACGTCCTGCACGACTCTAGAGAACCATCAGATGTTTCCAGGGTGCC  
CCAAGGACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATCAGTTCGCTT  
CTCGCTTCTGTTCGCGCGCTTCTGCTCCCCGAGCTCAATAAAAGAGCCCACAAC  
CCCTCACTCGG

The HREs can function in either orientation with respect to the promoter element.

30

OBHRE in combination with the MLV promoter

A series of vectors were constructed to analyse the activity of the HRE linked to the MoMLV promoter in a transient assay. The HRE and MoMLV promoter were removed from pLNheHRE as an *Nhe*I-*Sma*I fragment and inserted into the MCS of pGL3 basic (Promega) to produce pGLHRE and pGLMUT. PGL3 promoter and pGL3 control  
5 (Promega) were used as negative and positive controls respectively.

The vectors p5'HRE3'MUT, p5'MUT3'HRE, p5'MUT3'MUT and p5'HRE3'HRE were constructed by digesting pHRE and pMUT with *Sac*I and ligating the resulting retroviral genome with linearised pLNheHRE cut with the same enzyme.

10

The data shown (FIG3) clearly demonstrates that both 5' and 3' HRE are involved in transcriptional regulation and furthermore there is true synergy between the two. These data show that the optimum configuration of a hypoxia regulated vector is one where there is a duplication of the HRE in the 5' and 3' LTRs. This has lead us to design a regulated  
15 single transcription unit lentiviral vectors (see later).

It is important to note that these data can be extrapolated to other non HRE enhancer systems where a similar synergy can be envisaged.

20 By way of further example other promoters have also been constructed for use in the invention as shown in Figure 4a.

The relative strengths of these promoters are shown in Fig 4b. All these sequences confer on the minimal SV40 promoter hypoxia induced expression. However what is clear is the  
25 different induction ratios and the scale of expression under hypoxia.

Any of these promoter configurations can be used in the invention and the choice is dictated by the therapeutic gene. For example a highly toxic cytokine such as TNF-alpha would benefit from the use of the simple enolase promoter, as basal levels are undetectable  
30 in normoxia so guaranteeing that there is no inappropriate expression. A less toxic protein such as human cytochrome P450 that is needed in high levels would benefit from using

OBHRE1 where the maximum levels of expression are very high but the basal levels are detectable.

These promoters described above all contain DNA sequence motifs for the classical HRE binding transcription factor, HIF 1. This is a heterodimer consisting of HIF 1a and HIF 1b, a member of the basic helix-loop-helix (bHLH)/PAS domain protein family (from the founding members of this gene family; Period, ARNT, and SIM). HIF 1a was originally identified as a protein binding to the HRE of the erythropoietin gene in hepatoma cells (Wang et al 1995, J Biol Chem 270, 1230) but has since been implicated in the regulation of an expanding family of genes that are regulated by hypoxia in most cell types. Database searching of expressed cDNA sequences have identified a further bHLH/PAS family member that is closely related to HIF 1, termed EPAS-1 (endothelial PAS domain protein) or HRF (HIF-related factor) (Ema et al 1997, Proc Natl Acad Sci USA 94, 4273; Flamme et al 1997, Mech Dev 63, 51; Tian et al 1997, Genes Dev 11, 72). Unlike HIF 1, EPAS-1 is expressed predominantly in endothelial cells and its expression appears to be significantly regulated during development. As predicted from the conservation of the DNA binding domain of the two proteins, EPAS -1 appears to bind to the same consensus sequence (defined as the HRE) as HIF-1. Published data have however indicated that EPAS expression is restricted to endothelial cells. We now show (Figure 5) that, surprisingly, in macrophages it is not possible to detect the HIF-1 protein even under normoxia and yet the OBHRE promoter (PGK) is activated in these cells when they are placed in hypoxic conditions (Figure 6). We have analysed macrophages under the two conditions using antibodies that are specific for HIF-1 and EPAS. We could not detect HIF-1 in macrophages but we could detect EPAS. This is the first time that EPAS has been found outside of epithelial cells. Thus the induction of OB HRE 1 in macrophages is either principally or entirely mediated by EPAS-1 and as such represents an EPAS responsive enhancer.

It follows from these observations that there may be many factors, known or not yet discovered that mediate a transcriptional response under ischaemic conditions. Any piece

of DNA that is operationally responsive to such conditions irrespective of the factors involved is useful for the present invention.

Other conditions that are a consequence of ischaemia, such as low acidity or low glucose levels, can also be used to specifically activate genes. For example a promoter from the human *grp78* gene may be isolated by PCR amplification from human genomic DNA and includes the complete promoter-enhancer sequence and 5'UTR of the *grp78* gene. This is described in Chuck et al 1992, Nucleic Acids Res 20, 6481. The cloned fragment of 579 bp described in that paper corresponds to bases 6-585. The primers used in the amplification reaction incorporate an *AseI* site at the 5' end and a *XhoI* site at the 3' end. The *grp78* promoter fragment is then cloned into these sites present in a Clontech pEGFP-N1 vector allowing expression of a beta-gal/GFP fusion protein from this promoter.

#### Example 2. Construction of tissue restricted ischaemia responsive promoters

An alternative gene expression configuration confines the expression to cells that have differentiated into the granulocyte-monocyte lineage. Using promoters that are specific to, or upregulated in, the monocyte/macrophage lineage will restrict therapeutic gene expression to these cell types. Suitable promoters for this purpose include those with consensus sequences that bind myeloid specific transcription factors such as PU1 or transcription factors that are up-regulated during differentiation into macrophages such as CEBP- $\beta$  (NF IL 6) (See Clarke and Gordon 1998, J Leukoc Biol 63, 153).

These promoter elements can be isolated or synthesised by standard procedures. By way of example the isolation of two macrophage specific promoters derived from a cellular gene, and a viral gene is described. The HRE elements can be combined with any of these promoters. This is shown by way of example with the HIV derived promoter to produce a synthetic promoter referred to as XiaMac.

a) *Cellular promoter*

An oligonucleotide was synthesised in two parts based on tissue specific control elements residing in the M-CSF receptor promoter (Zhang et al 1994, Mol Cell Biol 14, 8085) flanked with a *HindIII/NcoI* site for cloning into pGL3 basic vector (Promega) and subsequent analysis in luciferase reporter assays. Sites of transcription factor binding are shown underneath.

AGCTTCCTGCCCCAGACTGCGACCCCTCCCTCTGGGTTCAAGGCTTTGTTTTCTT  
 10 SP1  
 CTTAAAGACCCAAGATTTCCAACTCTGTGGTTGCCTAGCTAAAAGGGGAA  
 CEBP? PEBP2/CBF PU. 1  
 GAAGAGGATCAGCCCAAGGAGGAC

b) *HIV promoter*

15 The transcription control sequences of HIV reside in the LTR, this generally consists of two NFkB sites and three SP1 sites upstream of the ATATAA box (Koong et al 1994, Cancer Res 54, 1425).

20 An Entrez database nucleotide search was performed for HIV LTR sequences and these were analysed for the integrity of the motifs outlined above. Submission U63538 (Zacharova et al 1997, AIDS Res Hum Retroviruses 13, 719) describes an isolate that shows the well conserved motifs of two NFkB sites and three SP1 sites. However we observed that this particular isolate also had the consensus sequence for binding of NF-IL6  
 25 (C/EBP $\beta$ ). As discussed above this transcription factor is known to be active in macrophages.

Oligonucleotides were synthesised in two parts to generate the sequence from Accession No U63538 positions 279 - 447. These were synthesised with *NheI/SmaI* ends to facilitate cloning. The annealed and ligated oligonucleotide was cloned into OB37.  
 30



The final construction is shown in Figure 7.

We have therefore constructed a novel synthetic promoter (XiaMac) that combines the 'classical' HRE mediated response with tissue specificity (macrophage). As shown in Figure 8 when the XiaMac promoter was tested in non-macrophage cells such as breast cancer cells there was no activity at all. However if the cells were subjected to hypoxia then activation was observed. In contrast the promoter was active in macrophage cell line and the activity was boosted by hypoxia. The XiaMac promoter response to hypoxia is abolished if the HRE is inactivated (Figure 9).

10

**Example 3. Construction of a macrophage specific promoter restricted by repression.**

An alternative promoter configuration is where sequences are added to further restrict the expression to macrophages under defined conditions.

15

An example of this approach would be the IRF system (Taniguchi et al 1995, J Cancer Res Clin Oncol 121, 516; Kuhl et al 1987, Cell 50, 1057). In this situation an interferon response element (IRE) can bind the transcription factors IRF1 and IRF 2. IRF 2 is constitutively bound to this in macrophages and lacks the ability to activate transcription. Interferon activates IRF 1 expression that can then compete for binding with IRF 2. The ability of IRF 1 to activate transcription thereby reverses IRF 2 repression. IRF 1 also induces IRF 2 gene expression thereby limiting activation of transcription by 'auto shut off'. Inclusion of a tetramer of the IRE can block SV40 promoter function in the absence of IRF 1 activation. Inclusion of this tetrameric sequence downstream of the HRE 5' to the ATATAA (i.e. the TATA box like element in the SV40 promoter) confers repression in the absence of activation by interferon.

Interferon gamma is naturally present in inflammatory responses including the response to tumours. Alternatively interferon gamma can be provided exogenously as a protein or as a gene and delivered as a gene therapy. In a particular aspect of the invention an autocrine regulatory circuit can provide interferon gamma. In this case a simple HRE promoter such

30

as OBHRE is linked to interferon gamma coding sequence. A second gene, for example a pro-drug activating enzyme or any from the above list is linked to the XiaMac promoter that contains an IRF-1 responsive sequence. The promoter is inactive in all cells including macrophages. Upon exposure to hypoxia in the pathological condition then interferon gamma is expressed. The expression of the therapeutic gene is then activated by the macrophage specific factors and the hypoxia responsive factors. This two phase strategy can be applied to any repressor protein. An over view of this strategy is shown in Figure 10.

10 The IRE is cloned into the XiaMac promoter as follows:-

Oligonucleotides are designed consisting of a tetramer of the IRE site as follows with BanII sticky ends:

15 C(AAGTGA)<sub>4</sub>GAGCC

Within the XiaMac promoter there is a BanII site which is 3' to the last SPI site and 15 bp upstream of the TATAA. Insertion of the IRE elements at this site produces a repressed promoter (XiaMac-IRE) that can only become active in the presence of interferon and hypoxia. This is shown in Figure 11.

#### Example 4. Construction of Endothelium specific promoters

The invention is not restricted to the generation of the macrophage lineage from HSC. For example the inclusion of an endothelium specific promoter restricts the expression of the therapeutic gene to vascular endothelium. In particular the correct choice of promoter can restrict expression to the neo-vasculature that is specific to tumours. For example Jaggar et al (1997, Hum Gene Ther 8 2239) have described the use of the e-selectin and KDR promoters to express therapeutic genes from retroviral vectors specifically in endothelial cells. We now show that these promoters can be configured into retroviral/lentiviral vectors and furthermore they can be additionally regulated by hypoxia. Configurations are shown

in Figure 12. These ILRE regulated endothelium specific promoters are particularly useful for the delivery of anti-angiogenic factors to tumour vasculature. The construction of lentiviral vectors is outlined in more detail in Example 6.

- 5 It follows that any promoter that restricts the expression to particular lineage derived from HSC can be used in combination with an ischaemia-like response element.

#### **Example 5. Construction of ILRE regulated retroviral vectors**

- 10 RRVs are constructed using a packaging cell line system such as FLYRD18 (Cosset et al 1995, J Virol 69, 7430). A plasmid vector containing the vector genome to be packaged is transfected into the packaging cell line as describe (Cosset et al 1995, J Virol 69, 7430) to derive the producer cell line. A suitable plasmid containing vector genome is pHIT111 (Soneoka et al 1995, Nucl Acids Res 23, 628). The required NOI is inserted in place of the  
15 LacZ gene in pHIT111 using standard molecular biology techniques. Regulatory elements such as HREs or a promoter-enhancer from the grp78 gene may similarly be introduced into the retroviral LTR in pHIT111 in place of the retroviral enhancer to ensure regulated expression of the NOI. The plasmid is then co-transfected with a selectable marker NOI appropriate for FLYRD18 cells (e.g. pSV2neo) and transfected cells are selected in 1  
20 mg/ml G418 (Sigma).

- A suitable HRE-containing enhancer (Fig 13) consists of three copies of the HRE from the PGK gene (Firth et al 1994, Proc Natl Acad Sci USA 91, 6496). Figure 13 also shows the sequence of a mutant HRE-containing sequence to be used as a control which is not  
25 regulated by hypoxia. The synthetic oligonucleotides shown in Figure 13 are inserted between the Nhe1 and Xba1 sites in the 3'LTR of pHIT111 to generate a retroviral vector in which gene expression in the target cell is under hypoxia control. An alternative retroviral vector, constructed in the same way, is pKAHRE shown in Figure 14.

- 30 Another configuration constructed according to the principals outlined above is found in Xia-Gen-P450-G (Figure 15a). This vector contains a therapeutic gene for human

cytochrome P450 which activates the anti-cancer compound cyclophosphamide. The CYP2B6 gene was obtained by PCR amplification from human hepatocyte derived mRNA. The correct gene sequence was confirmed by comparison to the established sequence (Yamano et al 1989 GenBank Accession No M29874). Any other therapeutic gene as  
5 recited above could be used. The RVV also contains a reporter gene that is green fluorescent protein and a selectable gene that is the neomycin resistance gene. The hypoxia mediated induction of this vector is shown by way of example in human tumour cells (Figure 15b).

#### 10 Example 6. Construction of hypoxia regulated lentiviral vectors

At present the state of the art in stem cell isolation and manipulation describes the preparation of cells that are probably dividing. It is generally accepted that the true stem cell which has the potential to develop into all lineages (toti-potent) is not dividing.  
15 Retroviral vectors will therefore miss these cells from a population of pluripotent CD34+ve cells. Lentiviral vectors offer significant advantages to conventional MLV based retroviral vectors because they can transfer genes to non-dividing and slowly dividing cells.

20 The hypoxia responsive promoter has been configured into a lentiviral vector, pEGASUS (Fig 16a) and the related vector pONY2.1 (Fig 16b). Both are derived from infectious proviral EIAV clone pSPEIAV19 (Payne et al 1998, J Virol 72, 483). The construction strategy is shown in the Figure 16 series. The CMV promoter in pONY 2.1 was excised as Xba1/Asc 1 fragment and replaced with an oligonucleotide containing a Mlu1/Xba 1 site.  
25 This consequently allows insertion of the Mlu/Xba fragment isolated from OB37 (Figure 2) creating pONY HRE luc/lac (Fig 16c). Luciferase coding sequence was removed as an Nco 1 fragment and the backbone religated creating pONY HRE lac. Similarly, lacZ was removed as Xba 1/ Sal1 then backbone religated to create pONY HRE luc. In the advanced EIAV vector plasmid pEGASUS the CMV promoter lacZ cassette was excised  
30 with EcoR1 and replaced with a synthetic oligonucleotide containing a SacII and a Bsu36 site. This allows the cloning of the HRE luc/HRE lac cassette from pONY 2.1 as

SacI/Bsu36 and SacI/EcoRI fragments respectively. The final vectors are designated pEG-HRE-lacZ (shown in Fig 16d), pEG-HRE-luc. Using the same approach pEGHRE vectors are constructed to express therapeutic genes in place of the lacZ or Luc genes.

- 5 Any of the therapeutic genes listed above are suitable. By way of example pEGHRE-TSP1 is shown. This expresses the anti-angiogenic factor thrombospondin-1. This is shown in Figure 17 in two typical configurations. A) is a silent LTR for use in cells where the ELAV LTR is largely inert. B) is a typical SIN (self-inactivating) vector where the U3 sequence is removed.
- 10 Viral particles with the pEGHRE genome are produced in a transient three plasmid system (Soneoka et al 1995, Nucl Acids Res 23, 628). Virus is titred on D17 cells on parallel plates and the plates are incubated overnight in normoxia (21% oxygen) or hypoxia (0.1% oxygen). Cells are stained by X gal histochemistry and end point titres calculated (see Figure 18). Titre is a measure of b-galactosidase gene expression and reflects changes in
- 15 gene expression between cell populations under conditions of normoxia and hypoxia.

- In the experiment shown the titre obtained from the parent 'pEGASUS' vector (with a CMV lacZ transcriptional unit) does not change significantly under hypoxia, whereas the regulated vector, pEG-HRE-lacZ, titre is induced by at least 100 fold. These data indicate
- 20 for the first time that it is possible to obtain highly efficient regulation in the context of a lentiviral vector

- Hypoxic regulation was also assessed with the luciferase reporter gene. This data reflects changes in promoter activity of cells within the population. Vector particles were produced
- 25 as outlined above and used to transduce D17 cells. The transduced cell population was split and incubated overnight in normoxia and hypoxia. Cells were processed for luciferase assay and luminometry. Luciferase activity of cells transduced with pEG-HRE-luc increases 12 fold under hypoxic conditions (Figure 19).

- 30 An alternative ELAV vector for use in this invention is where the therapeutic gene and a marker gene are expressed from a single transcription unit. Single transcription unit vectors

are generally preferred over SIN vectors and vectors containing internal transcription unit because of advantages in vector production. These advantages are that the SIN vector must be introduced into a producer cell by transfection rather than transduction and this often reduces the number of high efficiency producer clones that can be obtained. It is also observed that vectors that contain internal transcription units generally give lower yields than single transcription unit vectors. Furthermore as we describe above the duplication of the HRE that can be achieved in a single transcription unit vector optimises the regulation. A single transcription unit lentiviral vector regulated by hypoxia vector is described below:-

10

In this case the lacZ was isolated as a Xho 1-Sph1 fragment and cloned into pSP72 to make pSPLacZ. The IRES was generated by PCR from pIRES-1hyg (Clontech) to incorporate flanking restriction sites to enable subsequent cloning;

15

ST1 (lead)

ATCGCTCGAGCTGCAGGGCCGCACTAGAGGAATTCGC

20

ST2 (complement)

GGTTGTGGCCCATGGTATCATCGTGTTTTTCAAAGG

25

This results in a Xho1 IRES Nco1 cassette. This cassette is then cloned into pSPLacZ upstream of lacZ such that the Nco1 site coincides with the ATG initiator of lacZ, pSPIRESlacZ). The IRES lacZ cassette was isolated as a Pst1/Bst1107 fragment and used to replace the CMV lacZ cassette of pEIPEGASUS+ (excised as sse8387/Bst1107 fragment). This plasmid was designated pEG4+ IRES Z.

30

An overlapping PCR approach was used to replace part of the ELAV U3 enhancer region in the 3'LTR with an HRE. A similar vector can be constructed where the U3 region is largely derived from any retrovirus or lentivirus or and other hybrid promoter such as those described above. The only limitations are that the terminal nucleotides of the parental vector are retained to ensure compatability with the lentiviral integrase and that the two R regions are homologous to allow efficient reverse transcription. A typical configuration for

such a hybrid LTR is described in detail in PCT GB96/01230 and PCT/GB97/02858. The final configuration of this vector is shown in Figure 20.

5 Viral particles with the pEGHRE genome are produced either in a transient three plasmid system as described by Soneoka et al 1995, Nucl Acids Res 23, 628. Alternatively the vector can be introduced into a packaging cell line to make a stable producer cells. Suitable packaging lines that express the VSV-G envelope have been described [Yee et al. (1994 Proc. Natl. Acad. Sci. USA 91: 9564-9568; Ory et al. (1996 Proc. Natl. Acad. Sci. USA 93:11400-11406(Yang et al. (1995 Human Gene Therapy 6:1203-1213) Chen et al. (1996  
10 Proc. Natl. Acad. Sci. USA 93: 10057-10062 Lefkowitz et al., 1990, Virology 178;373-383).  
Arai et al. (1998 J. Virol. 72:1115-1121)))

15 Virus is titred on suitable indicator cells such as the dog cell line D17 cells on parallel plates and the plates are incubated overnight in normoxia (21% oxygen) or hypoxia (0.1% oxygen). Cells are stained by X gal histochemistry and end point titres calculated (see Figure 18). Titre is a measure of B-galactosidase gene expression and reflects changes in gene expression between cell populations under conditions of normoxia and hypoxia.

20 In the experiment shown the titre obtained from the parent pEGASUS vector (with a CMV lacZ transcriptional unit) does not change significantly under hypoxia, whereas the regulated vector, pEG-HRE-lacZ, titre is induced by at least 100 fold. These data indicate for the first time that it is possible to obtain highly efficient regulation in the context of a lentiviral vector

25 Hypoxic regulation was also assessed with the luciferase reporter gene. This data reflects changes in promoter activity of cells within the population. Vector particles were produced as outlined above and used to transduce D17 cells. The transduced cell population was split and incubated overnight in normoxia and hypoxia. Cells were processed for luciferase  
30 assay and luminometry. Luciferase activity of cells transduced with pEG-HRE-luc increases about 12 fold under hypoxic conditions (Figure 19).

### Construction of an autoregulated hypoxia responsive lenti-viral vector

It has previously been shown that the over expression of HIF-1 alpha can increase the expression from a number of hypoxia regulated promoters (Semenza et al JBC 1996.) This protein is known to be unstable in normal cells but this can be bypassed by over expression. Such over expression would therefore compromise the specificity of the hypoxia response. The discovery that the OBHRE promoter is responsive to E-PAS opens up a new route to amplifying the response to hypoxia. To avoid the problem of constitutive expression of HIF-1 we have configured the E-PAS gene into a lenti-viral vector that is itself responsive to hypoxia. In this way the vector is activated by hypoxia to express the E-PAS gene and the E-PAS protein then acts further on the HRE to augment expression. Given that HIF-1 is known to be labile once oxygen levels return to normal this vector system has the advantage of providing a longer term response to hypoxia. The initial priming of the vector is specific by virtue of the HRE interacting with HIF-1 or E-PAS. The production of E-PAS maintains the expression after the initial hypoxia stimulus is finished. Ultimately the response will decay according to the half life of the E-PAS protein.

The auto-regulated vector that expresses TSP-1 is shown in Figure 20b. The E-PAS coding sequence is obtained by PCR amplification using primers according to the known sequence of the cDNA (Accession number U81984; Triantafyllidis et al 1997, Gene Dev. 11, 72.)

#### Example 7. Construction of a hypoxia responsive adenoviral vector for the expression of a therapeutic gene.

When stem cells migrate to target tissue then they are still capable of delivering a therapy without division and differentiation, although such division is likely to occur at some stage. In this case vectors such as adenoviruses can be used. Such vectors do not integrate their genes into the cell genome and hence as the cell divides the vector is gradually lost from the cell population. However significant vector is still present in cells following



differentiation into various lineages. CD34<sup>+</sup> stem cells are transduced with recombinant adenoviral vectors according to a variety of methods. (Neering et al 1996 Blood 88, 1147; Watanabe et al 1996, Blood 87, 5032; Watanabe et al 1998, Leukaemia and Lymphoma 29, 439; Bregni et al 1998, Gene Ther 5, 465; Frey et al 1998, Blood 91, 2781).

5

A first generation recombinant adenoviral vector (E1/E3 deleted) has been constructed such that the bacterial  $\beta$ -galactosidase reporter gene is under the control of a hypoxically regulated promoter.

- 10 The first generation adenovirus vectors consist of a deletion of the E1 and E3 regions of the virus allowing insertion of foreign DNA, usually into the left arm of the virus adjacent to the left Inverted Terminal Repeat (ITR). The viral packaging signal (194-358nt) overlaps with the E1a enhancer and hence is present in most E1 deleted vectors. This sequence can be translocated to the right end of the viral genome (Hearing and Shenk 1983, Cell 33, 15 695). Therefore, in an E1 deleted vector 3.2 kb can be deleted (358-3525nt).

- Adenovirus is able to package 105% length of the genome, thus allowing for addition of an extra 2.1kb. Therefore, in an E1/E3 deleted viral vector the cloning capacity becomes 7 - 8 kb (2.1 kb +1.9 kb (removal of E3) and 3.2 kb (removal of E1). Since the recombinant 20 adenovirus lacks the essential E1 early gene it is unable to replicate in non-E1 complementing cell lines. The 293 cell line was developed by Graham et al (1977, J Gen Virol 36, 59) and contains approximately 4 kb from the left end of the Ad5 genome including the ITR, packaging signal, E1a, E1b and pIX. The cells stably express E1a and E1b gene products, but not the late protein IX, even though pIX sequences are within E1b. 25 In non-complementing cells the E1 deleted virus transduces the cell and is transported to the nucleus but there is no expression from the E1 deleted genome.

The diagram in Figure 21 shows the general strategy used to create recombinant adenoviruses using the Microbix Biosystems - NBL Gene Sciences system.

The general strategy involves cloning the foreign DNA into an E1 shuttle vector, where the E1 region from 402-3328 bp is replaced by the foreign DNA cassette. The recombinant plasmid is then co-transfected into 293 cells with the pJM17 plasmid. pJM17 contains a deletion of the E3 region and an insertion of the prokaryotic pBRX vector (including the ampicillin resistance and bacterial ori sequences) into the E1 region at 3.7 map units. This  
5 40 kb plasmid is therefore too large to be packaged into adeno nucleocapsids but can be propagated in bacteria. Intracellular recombination in 293 cells results in replacement of the amp<sup>r</sup> and ori sequences with the insert of foreign DNA.

10 In the examples quoted herein two transfer vectors have been used. The first obtained from Microbix is called pE1sp1A and the second obtained from Quantum Biotechnologies is called pADBN. The pADBN plasmid has the advantage that the new (foreign) DNA can be inserted in either orientation. This places the insert in a different spatial relationship with the resident adenoviral genes which can in some cases adversely affect expression. In  
15 both cases the second DNA is a defective version of the adenoviral genome, either as a plasmid for example pJM17 or as a part of the viral DNA for example the so-called right arm of Ad5. Homologous recombination generates the final gene transfer vector.

The construction of the ischaemia regulated adenoviral vector is described below:

20 The luciferase gene in OB37 (Figure 2) was replaced by the bacterial b-galactosidase encoding gene via an *Nco* I-*Xba* I fragment swap from the pONY2.1 vector (Figure 16b).

The resulting OB HRE LacZ cassette was removed from the OB37 vector as a *Kpn*I-*Sal*I fragment and cloned into the Quantum Biotechnologies™ pAdBN transfer vector producing AdenoOBHRElacZ.

25 The recombinant AdenoOBHRElacZ transfer vector was linearised (*Ase* I) and co-transfected into 293 cells along with the purified right arm of the Ad5 virus (from the *Cla* I site) to allow *in vivo* homologous recombination to occur resulting in the formation of the  
30 desired recombinant adenovirus. This is outlined in Figure 22. Adenoviral vectors

containing the HRE are referred to as AdHRE followed by the inserted gene, for example AdHRE-lacZ has the bacterial  $\beta$ -galactosidase gene expressed by the OBHRE promoter.

5 A range of different cell lines have been transduced with AdHRE-LacZ. After a 6 hour transduction the virus is removed and replaced with fresh medium and the cells are split into two separate plates for overnight incubation in either normoxia or hypoxia (0.1% oxygen). The results (Figure 23) demonstrate the hypoxic inducibility of the LacZ reporter gene within the adenoviral vector in Chiang Liver and the MCF-7 human breast cancer cell line.

10

In addition, 7-14 day old primary human macrophages have similarly been transduced with AdHRE-LacZ. This result not only demonstrates the transducibility but also the utility of using a HRE regulated recombinant adenovirus in cells in the haematopoietic lineage.

15 The inserted DNA construct present in the adenoviral transfer vector is in the form of an autonomous expression cassette containing the OBHRE promoter, the LacZ coding sequence and the SV40 polyadenylation signal (splice sites can also be included if necessary). In the system described for the construction of AdHRE (Quantum Biotech) we observed that a high level of protein expression was obtained if the expression cassette was  
20 directed in the orientation of the E1 genes.

Alternative hypoxia response elements may also be used as described earlier.

25 The HREs may be present in multiple copies both 5' and 3' to the gene to further increase the level of hypoxic induction.

In addition, the HRE could be combined with tissue specific promoter elements to restrict expression to specific tissue types or diseased tissue. For example, the OBHRE could be used in combination with the XiaMac promoters to regulate/increase expression  
30 specifically in macrophages.

AdHRE vectors have been configured to contain therapeutic genes.

An example is described below for the construction of AdHRE-2B6 and AdCMV-2B6 recombinant adenoviral vectors using the Microbix Biosystems construction system.

5 Plasmids are shown in Figure 24 and they are as follows:

**pE1HREPG** - The transfer vector engineered to contain the HRE driven 2B6 expression cassette

10 Using the E1sp1A transfer plasmid from Microbix the transfer vector pE1HREPG

The EMCV IRES GFP *Xba*I fragment from pCPGHRE is cloned into the *Xba*I site 3' to the 2B6 coding sequence in the pGL3OBHRE1p450 vector. The complete expression cassette is cloned into the Microbix transfer vector pΔE1sp1B as *Mlu*I-*Psh*AI fragment to  
15 give pE1HREPG (Figure 25b).

**pE1CMVPG** - The transfer vector engineered to contain the CMV driven 2B6 expression cassette (Figure 25c)

20 The BglII-NaeI CMV2B6 fragment from pCI-2B6 is cloned into the *Bam*HI-*Eco*RV site of pΔE1sp1B. The EMCV IRES GFP *Xba*I fragment from pCPGHRE is cloned into the *Xba*I site 3' to the 2b6 coding sequence in the resulting plasmid to create pE1CMVPG (Figure 25c).

Note: The use of the ires GFP reporter allows easier plaque purification of the recombinant  
25 adenovirus and provides viable cell marker for studying gene expression during different physiological conditions.

Any of the therapeutic genes outlined above can be inserted into the hypoxia regulated adenoviral vectors.

**Example 8. Construction of Adenoviral vectors to deliver lentiviral components**

In a particular aspect of the invention adenoviral vectors that contain the components that are required to make a retroviral or a lentiviral vector are used to transduce CD34+ve stem cells. The stem cell that has been transduced with the adenoviral vector therefore secretes the retroviral/lentiviral vector. The adenoviral vector in the CD34 cells therefore acts as an *in situ* retroviral factory as described previously [PCTGB97/00210]. The adenoviral vector can be configured in several ways. Firstly the expression of one or all of the retroviral/lentiviral vector components can be placed in a HRE regulated adenoviral vector as described above. Alternatively the vector components can be placed under a constitutive promoter such as the CMV promoter or a lineage specific promoter such as the e-selectin promoter or the macrophage specific promoter described above or a regulated promoter such as the tetracycline regulated system (Gosten and Bujard 1992 Proc Natl Acad Sci 89: 5547-5551) or any other regulated promoter/enhancer. In this latter case specificity is conferred by making the expression of the therapeutic gene from the retroviral/lentiviral vector regulated by hypoxia as described above. By way of example the production of an adenoviral vector that can produce a lentiviral vector is now described.

In order to produce lentiviral vectors four adenovirus vectors need to be made: genome, gagpol, envelope (for example rabies G) and Rev. The lentiviral components are expressed from heterologous promoters they contain introns where needed (for high expression of gagpol, Rev and Rabies envelope) and a polyadenylation signal. When these four viruses are transduced into E1a minus cells the adenoviral components will not be expressed but the heterologous promoters will allow the expression of the lentiviral components. An example is outlined below of the construction of an EIAV adenoviral system (Application number: 972135.7). The EIAV is based on a minimal system that is one lacking any of the non-essential EIAV encoded proteins (S2, Tat or envelope). The envelope used to pseudotype the EIAV is the rabies envelope (G protein). This has been shown to pseudotype EIAV well (Application number: 9811152.9).

## Transfer Plasmids

Described below is the construction of the transfer plasmids containing the EIAV  
5 components. The transfer plasmid is pE1sp1A (Figure 25a).

The recombinant transfer plasmids can then be used to make recombinant adenoviruses by  
homologous recombination in 293 cells.

10 A pictorial representation of the following plasmids is attached.

A) **pE1RevE.** This provides the Rev protein required for the efficient expression  
of gag and pol

15 The plasmid pCI-Rev is cut with Apa I and Cla I. The 2.3 kb band encoding EIAV Rev  
is blunt ended with Klenow polymerase and inserted into the Eco RV site of pE1sp1A to  
give plasmid pE1RevE (Figure 26).

B) **pE1HORSE3.1-gagpol Construct**

20 pHORSE3.1 was cut with Sna BI and Not I. The 6.1 kb band encoding EIAV gagpol was  
inserted into pE1RevE cut with Sna BI and Not I (7.5 kb band was purified). This gives  
plasmid pE1HORSE3.1 (Figure 27).

25 C) **pE1PEGASUS-Genome Construct**

pEGASUS4 was cut with Bgl II and Not I. The 6.8 kb band containing the EIAV vector  
genome was inserted into pE1RevE cut with Bgl II and Not I (6.7 kb band was purified).  
This gave plasmid pE1PEGASUS (Figure 28).

**D) pCI-Rab - Rabies Construct**

5 In order to make the pE1Rab the rabies open reading frame was inserted into pCI-Neo (Figure 29) by cutting plasmid pSA91RbG with Nsi I and Ahd I. The 1.25 kb band was bluntended with T4 DNA polymerase and inserted into pCI-Neo cut with Sma I. This gave plasmid pCI-Rab (Figure 30).

**E) pE1Rab - Rabies Construct**

10 pCI-Rab was cut with Sna BI and Not I. The 1.9kb band encoding Rabies envelope was inserted into pE1RevE cut with Sna BI and Not I (7.5 kb band was purified). This gave plasmid pE1Rab (Figure 31).

15 Any therapeutic gene or combination of genes can be inserted into the lentiviral vector as described above.

**Example 9. Engineering stem cells to express a prodrug activating enzyme in response to hypoxia.**

20 The retroviral vector, XiaGen-P450-G or the lentiviral vector pSec-TSp1 is used to transduce any cell type. As an example, we use human haematopoietic stem cells, human peripheral blood buffy coat and human cord blood. Procedures for isolation of CD34<sup>+</sup> HSCs and retroviral mediated gene transfer into these cells are described (such as Charbord  
25 et al 1996, Br J Haematol 94, 449; Dunbar et al 1996, Hum Gene Ther 7, 231; Cassel et al 1993, Exp Haematol 21, 585; Emmons et al 1997, Blood 89, 4040; Jolly et al 1996 WO 96/33281; Kerr et al WO96/09400). An example of a suitable method is as follows.

30 HSCs are harvested from peripheral blood after mobilisation with G-CSF and/or cyclophosphamide (Casset et al 1993, Exp Haematol 21, 585). G-CSF (Amgen) is given at a dose of 10 µg/kg/day sub-cutaneously for 7 days. Apheresis and enrichment of HSCs is

carried out using the CellPro Stem Cell Separator system (Cassel et al 1993, Exp Haematol 21, 585). The HSC-enriched population is cultured at  $10^5$  cells/ml in spent medium from RRV producer cells (Example 1) in the presence of 4  $\mu$ g/ml protamine sulphate and 20 ng/ml IL-3 (Sandoz), 50 ng/ml IL-6 (Sandoz), 100 ng/ml SCF (Amgen) (Santiago-Schwartz et al 1992, J Leuk Biol 52, 274; Charbord et al 1996, Br J Haematol 94, 449; Dao et al 1997, Blood 89, 446; Piacibello et al 1997, Blood 89, 2644; Cassel et al 1993, Exp Haematol 21, 585). Other cytokines and/or autologous stromal cells prepared as described (Dunbar et al 1996, Hum Gene Ther 7, 231) may also be added. After 24 hours the cells are centrifuged and resuspended in fresh RRV-containing medium with growth factors and protamine sulphate as above. This is repeated after a further 24 hours and the cells cultured for up to a further 48 hours. After this time the cells are trypsinised, washed several times in fresh medium by centrifugation and resuspended in Plasma-Lyte A for re-infusion. The total volume for re-infusion is approximately 25 - 50ml. Patients are infused over a period of up to two hours. The number of cells infused is at least  $10^5$  cells and may be up to the order of  $10^{12}$  cells.

Cells may also be matured along the myeloid differentiation pathway prior to re-infusion according to published methods (Haylock et al 1992, Blood 80, 1405).

Transduction is as follows as described for retroviral vectors but the same methods are used for lentiviral vectors:-

Gene transfer with retroviral or lentiviral vectors has been enhanced by optimising culture conditions of isolated CD34 cells such that they are cycling at the time of transduction. This is particularly necessary with MLV based vectors that require cell division to enable nuclear access and integration. Pseudotyping MLV vectors with different envelopes has also had a major impact on gene transfer. As reported in the literature GALV is markedly better than VSVG.

We have used a defined medium containing to ensure that cells are cycling at the time of transduction. Use of a defined media ensures that proliferative factors are present in the



absence of anti-proliferative factors that may be found in serum or more complex media.

Upon isolation CD34 cells are transferred to the following media (based principally on Becker et al 1998, Hum Gene Ther 9, 1561) for at least 24 hours prior to transduction. Serum-free medium X-VIVO 10, 1% BSA, 2mM L-glutamine, 1% pen/strep, 20ng/ml IL 3, 100U/ml IL 6, 50ng/ml SCF, 100 ng/ml anti-TGFb, 100 ng/ml Flt 3-L. The transduction protocol outlined (Becker et al 1998 HGT 9 1561-1570) is used. This involves three cycles over 3 days and optimises the likelihood of the majority of cells undergoing mitosis in the presence of viral vector. The method is briefly as follows:-

Coat non-tissue culture grade plates with fibronectin fragment CH-269 at  $10\mu\text{g}/\text{cm}^2$  (Takahara). Add virus to empty wells and allow to bind for 30mins. Wash with PBS. Add  $10^5$  cells in 0.5ml media per well. Add 0.5ml virus of supernatant. Centrifuge at 1020xg for 90min. After 4 hours replace with fresh media as above. This is repeated for 3 consecutive days.

Cells isolated from peripheral blood or from sources derived from peripheral blood preferentially form a higher proportion of erythroid progenitors whereas CD34 cells from cord blood results in the formation of a broader spread of progenitor type. Cord blood derived cells have a higher inherent proliferative capacity. Increased stem cell numbers can be derived from peripheral blood that has been 'mobilised' prior to isolation with GM-CSF. This approach is widely used in transplant patients.

Cells are then plated out in methocel medium containing growth factors (Methocel containing 10% fetal bovine serum, transferrin, glutamine, 2-mercaptoethanol, bovine serum albumin, IL3, IL6, IL11, SCF, Epo, GCSF, GMCSF) in a dark Class II safety cabinet according to the following protocol. Using a blunt needle attached to a 2.5ml syringe, dispense 2.5ml methocel into a 10ml U tube avoiding air bubbles. Dispense 0.5ml of the cell suspension (containing 3x the cell number required for each well so that 1ml of the methocel will contain the desired cell number of 500/2000 cells per well) in Iscove's medium (Gibco) into the tube containing the methocel and vortex gently. Gently dispense

1ml of the methocel and cell mix into a non-tissue culture petri dish (35x10mm) or a single well coverglass chamber using a blunt needle and syringe. Triplicate dishes/wells can be set up from each sample. Place the dishes/chambers into a Falcon 3025 dish (150x25mm) housing small petri dishes of sterile distilled water to ensure correct humidity. Incubate  
5 dishes at 37°C, 5% CO<sub>2</sub> and 5% O<sub>2</sub> and analyse colonies after 14 days.

To enhance production of CD34 derived monocytes, culture conditions are phased to optimise formation of GM progenitors (Haylock et al 1992, Blood 80, 1405) and subsequently monocyte progenitors. This is enabled using the CFU assay to monitor aliquots from the primary culture and ensure conditions are optimal for monocyte  
10 generation. Supplementation of culture media with relatively high doses of GM CSF to optimise generation of GM progenitors, followed by phased administration of MCSF as the dominant growth factor skews differentiation toward the myeloid lineage. Following transduction of the HSC they are differentiated to form GM precursors

15 The GM precursor colonies are analysed for the expression of the retroviral vector by observation of the fluorescence of GFP. This is assessed under normoxia and hypoxia.

Alternatively the stem cell population can be transduced using the same procedures as described above but immediately after isolation. They can then be frozen or used  
20 immediately for transfer into patients. In this case totipotent stem cells are preserved.

#### **Example 10. Interaction of engineered HSCs with ovarian cancer xenografts**

As an example of the invention we have chosen to study HSC infiltration into tumours  
25 using ovarian cancer as the test system. This cancer is largely confined to the peritoneal cavity. A number of animal models are available to analyse HSC infiltration into ovarian tumours. Three human ovarian cancer xenografts OS, HU and LA have been derived from primary human tumours and established intraperitoneally and they are maintained by serial passage by that route (Ward et al 1987, Cancer Res 47, 2662). None of these lines grow  
30 well in long term culture. All grow primarily as free floating ascites surrounded by mucin. This is not reflective of the human disease that exists as solid implanted tumour deposits

with accompanying ascites. Intraperitoneal administration of TNF converts the HU and LA models from this ascitic state to implanted solid tumour deposits on the surface of the peritoneum and associated organs (Malik et al 1989, Int. J. Cancer 44, 918). This closely represented the pathology of the human disease and more specifically resembles the original pathology of the tumour from which they were derived. This solid tumour model has been used as a more relevant starting point for study of therapeutic agents in ovarian cancer. (eg. interferon gamma, Malik et al 1991, Cancer Res 51, 6643; Burke et al 1997, Eur. J. Cancer, 33 1114). Human stem cells engineered with the adenoviral or retroviral or lentiviral vectors described above are introduced at a concentration of  $10^6$  /0.1ml into the peritoneal cavity of mice bearing the tumours described above. The HSCs express the therapeutic genes. The action of these genes extends beyond the HSC i.e. there is a bystander effect so that the surrounding tumour tissue is killed. In a particular example of this invention the vector XiaGen-P450 is used. The mice are treated with the engineered HSC and then treated with cyclophosphamide at 100mg/Kg administered intraperitoneally. The tumours of mice treated with engineered HSC are killed by the cyclophosphamide.

Similar procedures are used with AdHRE vectors following the published stem cell transduction procedure as follows. Adenoviral vector is used to transduce HSC at a high multiplicity of infection (100 to 500) in a small volume of culture medium containing 200 units/ml IL-3, 200 units/ml GM-CSF, 200 units/ml G-CSF for 24 hours. After transduction with the adenoviral vector the HSC can either be differentiated or used directly. If differentiated cells are to be used then colony forming units-granulocyte/macrophage (CFU-GM) are quantified in soft agar containing the above cytokines after incubation for 14 days. The CD34+ cells or differentiated cells are introduced into the animal and migrate to the target tissue and express a therapeutic gene.

#### **Example 11. Treatment of ovarian cancer with modified human stem cells**

Peripheral blood lymphocytes are collected from ovarian cancer patients as follows. The cells are transduced with the XiaGen-P450 retroviral vector as described above. The engineered stem cells are returned to the patient via an intraperitoneal injection broadly as

described for monocytes by Stevenson et al 1987, Cancer Res 47, 6100. Basically  $10^8$  to  $10^9$  cells in 50mls of isotonic saline are introduced directly into the abdominal cavity via a needle catheter monitored by ultrasound. The cells are distributed widely in the peritoneal cavity and remain associated with serosal surfaces and do not traffic to other organs. After 1 to 7 days the patients are treated with cyclophosphamide and tumours are analysed after a further 2 weeks. Reduction in tumour mass is a consequence of local activation of the cyclophosphamide by the engineered stem cells.

Similar procedures are used with AdHRE vectors following the stem cell transduction procedure outline above.

#### **Example 12. Transduction of tumour cells with lenti-viral vectors**

As a further example of the present invention we have chosen to study gene transfer to glioma. Gliomas are characterised by hypervascularity and invasiveness yet they are one of the most hypoxic tumours that have been studied [J. Folkman pp3075- 3085 In Cancer: Principles and Practice of Oncology, Fifth Edition ed DeVita, Hellman, Rosenberg. Pub. Lippincott-Raven 1997]. A lentiviral vector is configured to deliver a combination therapy of a prodrug activating enzyme and an anti angiogenic factor, in this example this is the human cytochrome P450 gene and the anti-angiogenic factor TSP-1. The expression is activated in hypoxia thus ensuring that the therapy is administered in the local environment of the tumour. A suitable lentiviral vector is described in the Figure 32. The model systems use either the human cell line U87MG or the rat RT-2 line. Both give typical vascularised tumours that can be analysed either in normal rats or in nude or SCID mice (e.g. Wei et al 1994, Human Gene Therapy 5, 969). In both cases the tumour model is created by the intra-cerebral implantation of tumour cell lines. Lentiviral vector preparations are injected directly into the tumour mass at multiple sites. Vector is delivered in 1 to 3 ul amounts at a titre of at least  $10^4$  transducing units/ul. The same procedure is used in treating human patients. In this case the tumour is located by PET or MRI scanning and injected with vector in 0.1ml aliquots or alternatively at the time of surgical debulking the site can be treated with vector. Patients are treated with cyclophosphamide and the reduction in tumour growth is monitored by MRI scanning.

**Example 13. Induction of lentiviral vector production and expression by desferrioxamine**

5 Desferrioxamine is obtained from Sigma or as a clinical formulation from Novartis Pharmaceuticals as the licensed product Deferal. The level of induction achieved with Desferal is equal to or greater than that achieved by hypoxia.

10 For in vitro use producer cells containing the hypoxia regulated lentiviral vectors are cultured in flasks for 10 days in the presence of 50 micromolar to 1 millimolar desferrioxamine. Cultures release vector particles during this period to give total yields in excess of  $10^7$ /ml. For scale up cells are cultured in roller bottles and desferrioxamine is used at 50 to 200 micromolar for seven days. This system therefore exploits the presence of the HRE to allow induction of viral vectors. The system is described whereby the genome is  
15 regulated by HRE in response to desferrioxamine. It follows that the other components i.e. the gagpol and the envelope can be similarly regulated. It follows that this system can be used to regulate the production of the components from any retroviral or lentiviral vector.

For in vivo use patients are treated with the hypoxia regulated lentiviral vector or with cells  
20 that contain the hypoxia regulated lentiviral vector. Patients are then given a standard course of treatment with desferrioxamine. This activates the therapy in addition to any effects of hypoxia and in some cases may replace the requirement for local hypoxia. For example if the cells are implanted to provide a therapeutic protein such as Epo or a blood clotting factor, such as factor IX then the delivery can be regulated by adjusting the dose of  
25 Desferal.

**Split Intron Technology**

The following teachings are taken from our co-pending application and provide teachings  
30 on how to devise retroviral vectors with split-intron features. These teachings can be adapted to prepare one or more vectors that are capable of delivering ILRE regulated NOIs

to cells in combination with a split-intron configuration. That PCT patent application is annexed hereto and all of its contents are incorporated herein by reference.

### Summary

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In a broad aspect the present invention provides a modified cell comprising a response element that is active in that cell; wherein the modified cell is prepared by transforming a cell or progenitor cell therefor by viral transduction with one or more viral vectors wherein at least one of which comprises the response element.

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In a preferred aspect the present invention provides a modified cell comprising a response element that is active in that cell; wherein the modified cell is prepared by transforming a cell or progenitor cell therefor by viral transduction with one or more viral vectors wherein at least one of which comprises the response element, and wherein the response element

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comprises an ILRE.

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In a preferred aspect the present invention provides a modified cell comprising a response element that is active in that cell; wherein the modified cell is prepared by transforming a cell or progenitor cell therefor by viral transduction with one or more viral vectors wherein at least one of which comprises the response element, and wherein the response element comprises an HRE.

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In a more preferred aspect the present invention provides a modified haematopoietic stem cell (MHSC) comprising at least one expressable nucleotide sequence of interest (NOI) wherein the or each NOI is operably linked to one or more response elements comprising an ischaemia like response element (ILRE).

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The vectors, constructs, regulatory elements and promoters described in the Examples Section - each of which is novel - are also encompassed by the present invention.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred  
5 embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

## ANNEX

## VECTOR

The present invention relates to a vector.

- 5 In particular, the present invention relates to a novel system for packaging and expressing genetic material in a retroviral particle.

More in particular, the present invention relates to a novel system capable of expressing a retroviral particle that is capable of delivering a nucleotide sequence of interest  
10 (hereinafter abbreviated as "NOI") - or even a plurality of NOIs - to one or more target sites.

In addition, the present invention relates to *inter alia* a novel retroviral vector useful in gene therapy.

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Gene therapy may include any one or more of: the addition, the replacement, the deletion, the supplementation, the manipulation etc. of one or more nucleotide sequences in, for example, one or more targeted sites - such as targeted cells. If the targeted sites are targeted cells, then the cells may be part of a tissue or an organ.

- 20 General teachings on gene therapy may be found in Molecular Biology (Ed Robert Meyers, Pub VCH, such as pages 556-558).

By way of further example, gene therapy can also provide a means by which any one or more of: a nucleotide sequence, such as a gene, can be applied to replace or supplement  
25 a defective gene; a pathogenic nucleotide sequence, such as a gene, or expression product thereof can be eliminated; a nucleotide sequence, such as a gene, or expression product thereof, can be added or introduced in order, for example, to create a more favourable phenotype; a nucleotide sequence, such as a gene, or expression product thereof can be added or introduced, for example, for selection purposes (i.e. to select  
30 transformed cells and the like over non-transformed cells); cells can be manipulated at the molecular level to treat, cure or prevent disease conditions - such as cancer (Schmidt-



Wolf and Schmidt-Wolf, 1994, *Annals of Hematology* 69;273-279) or other disease conditions, such as immune, cardiovascular, neurological, inflammatory or infectious disorders; antigens can be manipulated and/or introduced to elicit an immune response, such as genetic vaccination.

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In recent years, retroviruses have been proposed for use in gene therapy. Essentially, retroviruses are RNA viruses with a life cycle different to that of lytic viruses. In this regard, a retrovirus is an infectious entity that replicates through a DNA intermediate. When a retrovirus infects a cell, its genome is converted to a DNA form by a reverse transcriptase enzyme. The DNA copy serves as a template for the production of new RNA genomes and virally encoded proteins necessary for the assembly of infectious viral particles.

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There are many retroviruses and examples include: murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV).

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A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

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Details on the genomic structure of some retroviruses may be found in the art. By way of example, details on HIV may be found from the NCBI Genbank (i.e. Genome Accession No. AF033819).

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Essentially, all wild type retroviruses contain three major coding domains, *gag*, *pol*, *env*, which code for essential virion proteins. Nevertheless, retroviruses may be broadly

divided into two categories: namely, "simple" and "complex". These categories are distinguishable by the organisation of their genomes. Simple retroviruses usually carry only elementary information. In contrast, complex retroviruses also code for additional regulatory proteins derived from multiple spliced messages.

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Retroviruses may even be further divided into seven groups. Five of these groups represent retroviruses with oncogenic potential. The remaining two groups are the lentiviruses and the spumaviruses. A review of these retroviruses is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 1-25).

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All oncogenic members except the human T-cell leukemia virus-bovine leukemia virus group (HTLV-BLV) are simple retroviruses. HTLV, BLV and the lentiviruses and spumaviruses are complex. Some of the best studied oncogenic retroviruses are Rous sarcoma virus (RSV), mouse mammary tumour virus (MMTV) and murine leukemia virus (MLV) and the human T-cell leukemia virus (HTLV).

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The lentivirus group can be split even further into "primate" and "non-primate". Examples of primate lentiviruses include the human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

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A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells (Lewis *et al* 1992 EMBO. J 11: 3053-3058; Lewis and Emerman, 1994 J. Virol. 68: 510-516). In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue.

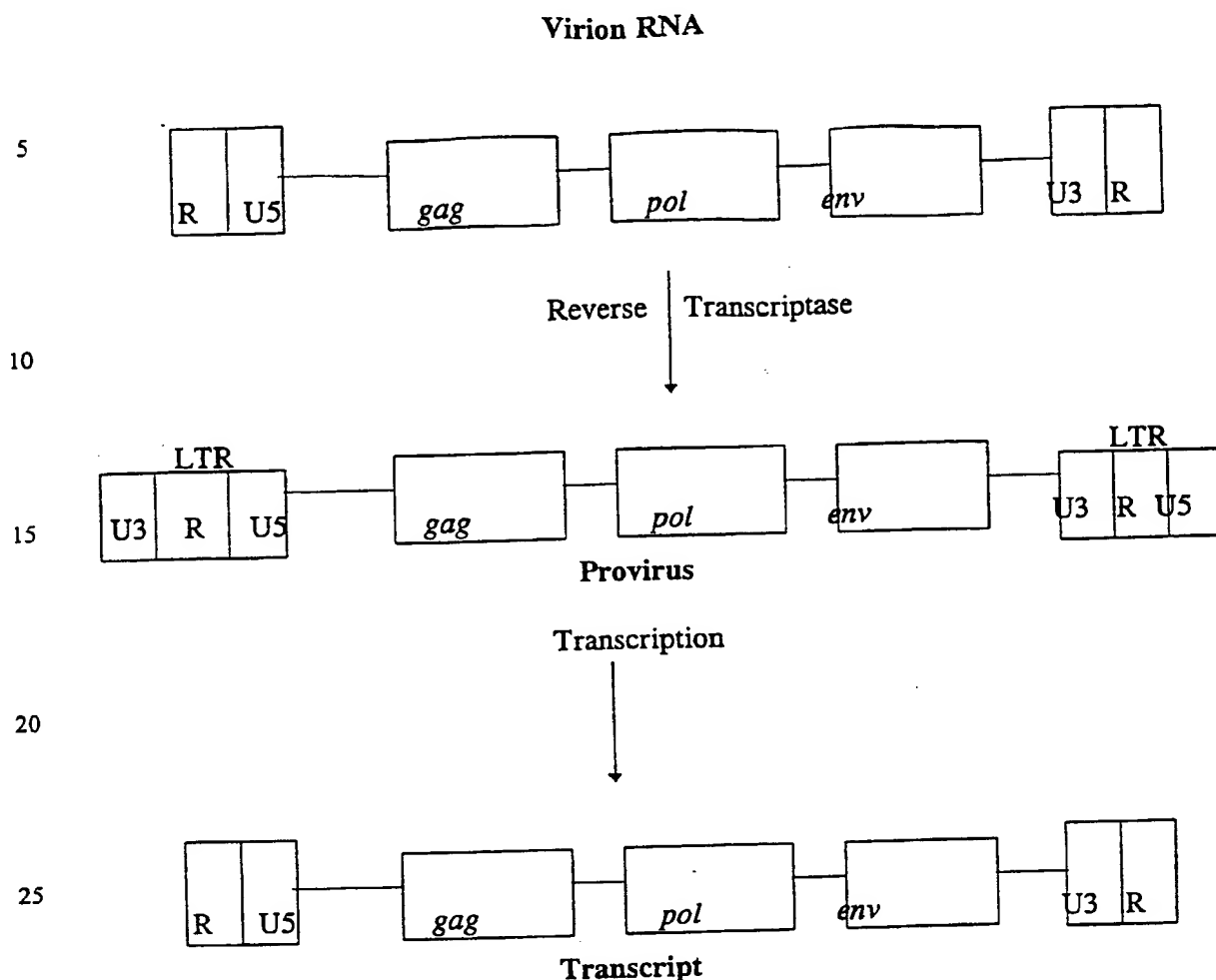
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During the process of infection, a retrovirus initially attaches to a specific cell surface receptor. On entry into the susceptible host cell, the retroviral RNA genome is then copied to DNA by the virally encoded reverse transcriptase which is carried inside the parent virus. This DNA is transported to the host cell nucleus where it subsequently integrates into the host genome. At this stage, it is typically referred to as the provirus. The provirus is stable in the host chromosome during cell division and is transcribed like other cellular proteins. The provirus encodes the proteins and packaging machinery required to make more virus, which can leave the cell by a process sometimes called "budding".

As already indicated, each retroviral genome comprises genes called *gag*, *pol* and *env* which code for virion proteins and enzymes. In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. They also serve as enhancer-promoter sequences. In other words, the LTRs can control the expression of the viral gene. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence located at the 5' end of the viral genome.

The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

For ease of understanding, simple, generic structures (not to scale) of the RNA and the DNA forms of the retroviral genome are presented below in which the elementary features of the LTRs and the relative positioning of *gag*, *pol* and *env* are indicated.



As shown in the diagram above, the basic molecular organisation of an infectious retroviral RNA genome is (5') R - U5 - *gag*, *pol*, *env* - U3-R (3'). In a defective retroviral vector genome *gag*, *pol* and *env* may be absent or not functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.

Reverse transcription of the virion RNA into double stranded DNA takes place in the cytoplasm and involves two jumps of the reverse transcriptase from the 5' terminus to the 3' terminus of the template molecule. The result of these jumps is a duplication of sequences located at the 5' and 3' ends of the virion RNA. These sequences then occur fused in tandem on both ends of the viral DNA, forming the long terminal repeats

(LTRs) which comprise R U5 and U3 regions. On completion of the reverse transcription, the viral DNA is translocated into the nucleus where the linear copy of the retroviral genome, called a preintegration complex (PIC), is randomly inserted into chromosomal DNA with the aid of the virion integrase to form a stable provirus. The number of possible sites of integration into the host cellular genome is very large and very widely distributed.

The control of proviral transcription remains largely with the noncoding sequences of the viral LTR. The site of transcription initiation is at the boundary between U3 and R in the left hand side LTR (as shown above) and the site of poly (A) addition (termination) is at the boundary between R and U5 in the right hand side LTR (as shown above). U3 contains most of the transcriptional control elements of the provirus, which include the promoter and multiple enhancer sequences responsive to cellular and in some cases, viral transcriptional activator proteins. Some retroviruses have any one or more of the following genes such as *tat*, *rev*, *tax* and *rex* that code for proteins that are involved in the regulation of gene expression.

Transcription of proviral DNA recreates the full length viral RNA genomic and subgenomic-sized RNA molecules that are generated by RNA processing. Typically, all RNA products serve as templates for the production of viral proteins. The expression of the RNA products is achieved by a combination of RNA transcript splicing and ribosomal frameshifting during translation.

RNA splicing is the process by which intervening or "intronic" RNA sequences are removed and the remaining "exonic" sequences are ligated to provide continuous reading frames for translation. The primary transcript of retroviral DNA is modified in several ways and closely resembles a cellular mRNA. However, unlike most cellular mRNAs, in which all introns are efficiently spliced, newly synthesised retroviral RNA must be diverted into two populations. One population remains unspliced to serve as the genomic RNA and the other population is spliced to provide subgenomic RNA.

competing splice acceptors can cause shifts in the splicing patterns and can affect viral infectivity (Purcell and Martin 1993 J Virol 67: 6365-6378).

5 The relative proportions of full-length RNA and subgenomic-sized RNAs vary in infected cells and modulation of the levels of these transcripts is a potential control step during retroviral gene expression. For retroviral gene expression, both unspliced and spliced RNAs must be transported to the cytoplasm and the proper ratio of spliced and unspliced RNA must be maintained for efficient retroviral gene expression. Different classes of retroviruses have evolved distinct solutions to this problem. The simple  
10 retroviruses, which use only full-length and singly spliced RNAs regulate the cytoplasmic ratios of these species either by the use of variably efficient splice sites or by the incorporation of several *cis*-acting elements, that have been only partially defined, into their genome. The complex retroviruses, which direct the synthesis of both singly and multiply spliced RNA, regulate the transport and possibly splicing of the different  
15 genomic and subgenomic-sized RNA species through the interaction of sequences on the RNA with the protein product of one of the accessory genes, such as *rev* in HIV-1 and *rex* in HTLV-1.

With regard to the structural genes *gag*, *pol* and *env* themselves and in slightly more  
20 detail, *gag* encodes the internal structural protein of the virus. Gag is proteolytically processed into the mature proteins MA (matrix), CA (capsid) and NC (nucleocapsid). The *pol* gene encodes the reverse transcriptase (RT), which contains both DNA polymerase, and associated RNase H activities and integrase (IN), which mediates replication of the genome. The *env* gene encodes the surface (SU) glycoprotein and the  
25 transmembrane (TM) protein of the virion, which form a complex that interacts specifically with cellular receptor proteins. This interaction leads ultimately to fusion of the viral membrane with the cell membrane.

The Env protein is a viral protein which coats the viral particle and plays an essential  
30 role in permitting viral entry into a target cell. The envelope glycoprotein complex of retroviruses includes two polypeptides: an external, glycosylated hydrophilic polypeptide

(SU) and a membrane-spanning protein (TM). Together, these form an oligomeric "knob" or "knobbed spike" on the surface of a virion. Both polypeptides are encoded by the *env* gene and are synthesised in the form of a polyprotein precursor that is proteolytically cleaved during its transport to the cell surface. Although uncleaved Env proteins are able to bind to the receptor, the cleavage event itself is necessary to activate the fusion potential of the protein, which is necessary for entry of the virus into the host cell. Typically, both SU and TM proteins are glycosylated at multiple sites. However, in some viruses, exemplified by MLV, TM is not glycosylated.

Although the SU and TM proteins are not always required for the assembly of enveloped virion particles as such, they play an essential role in the entry process. In this regard, the SU domain binds to a receptor molecule, often a specific receptor molecule, on the target cell. It is believed that this binding event activates the membrane fusion-inducing potential of the TM protein after which the viral and cell membranes fuse. In some viruses, notably MLV, a cleavage event, resulting in the removal of a short portion of the cytoplasmic tail of TM, is thought to play a key role in uncovering the full fusion activity of the protein (Brody *et al* 1994 J Virol 68: 4620-4627; Rein *et al* 1994 J Virol 68: 1773-1781). This cytoplasmic "tail", distal to the membrane-spanning segment of TM remains on the internal side of the viral membrane and it varies considerably in length in different retroviruses.

Thus, the specificity of the SU/receptor interaction can define the host range and tissue tropism of a retrovirus. In some cases, this specificity may restrict the transduction potential of a recombinant retroviral vector. Here, transduction includes a process of using a viral vector to deliver a non-viral gene to a target cell. For this reason, many gene therapy experiments have used MLV. A particular MLV that has an envelope protein called 4070A is known as an amphotropic virus, and this can also infect human cells because its envelope protein "docks" with a phosphate transport protein that is conserved between man and mouse. This transporter is ubiquitous and so these viruses are capable of infecting many cell types. In some cases however, it may be beneficial, especially from a safety point of view, to specifically target restricted cells. To this end, several groups have engineered a mouse ecotropic retrovirus, which unlike its

amphotropic relative normally only infects mouse cells, to specifically infect particular human cells. Replacement of a fragment of an Env protein with an erythropoietin segment produced a recombinant retrovirus which then binds specifically to human cells that express the erythropoietin receptor on their surface, such as red blood cell precursors (Maulik and Patel 1997 "Molecular Biotechnology: Therapeutic Applications and Strategies" 1997 Wiley-Liss Inc. pp 45).

In addition to *gag*, *pol* and *env*, the complex retroviruses also contain "accessory" genes which code for accessory or auxillary proteins. Accessory or auxillary proteins are defined as those proteins encoded by the accessory genes in addition to those encoded by the usual replicative or structural genes, *gag*, *pol* and *env*. These accessory proteins are distinct from those involved in the regulation of gene expression, like those encoded by *tat*, *rev*, *tax* and *rex*. Examples of accessory genes include one or more of *vif*, *vpr*, *vpx*, *vpu* and *nef*. These accessory genes can be found in, for example, HIV (see, for example pages 802 and 803 of "Retroviruses" Ed. Coffin *et al* Pub. CSHL 1997). Non-essential accessory proteins may function in specialised cell types, providing functions that are at least in part duplicative of a function provided by a cellular protein. Typically, the accessory genes are located between *pol* and *env*, just downstream from *env* including the U3 region of the LTR or overlapping portions of the *env* and each other.

The complex retroviruses have evolved regulatory mechanisms that employ virally encoded transcriptional activators as well as cellular transcriptional factors. These *trans*-acting viral proteins serve as activators of RNA transcription directed by the LTRs. The transcriptional *trans*-activators of the lentiviruses are encoded by the viral *tat* genes. Tat binds to a stable, stem-loop, RNA secondary structure, referred to as TAR, one function of which is to apparently optimally position Tat to *trans*-activate transcription.

As mentioned earlier, retroviruses have been proposed as a delivery system (otherwise expressed as a delivery vehicle or delivery vector) for *inter alia* the transfer of a NOI, or a plurality of NOIs, to one or more sites of interest. The transfer can occur *in vitro*, *ex*



*vivo*, *in vivo*, or combinations thereof. When used in this fashion, the retroviruses are typically called retroviral vectors or recombinant retroviral vectors. Retroviral vectors have even been exploited to study various aspects of the retrovirus life cycle, including receptor usage, reverse transcription and RNA packaging (reviewed by Miller, 1992  
5 Curr Top Microbiol Immunol 158:1-24).

In a typical recombinant retroviral vector for use in gene therapy, at least part of one or more of the *gag*, *pol* and *env* protein coding regions may be removed from the virus. This makes the retroviral vector replication-defective. The removed portions may even  
10 be replaced by a NOI in order to generate a virus capable of integrating its genome into a host genome but wherein the modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in, for example, a therapeutic and/or a diagnostic effect. Thus, the transfer of a NOI into a site of interest is typically achieved by: integrating the NOI into  
15 the recombinant viral vector; packaging the modified viral vector into a virion coat; and allowing transduction of a site of interest - such as a targeted cell or a targeted cell population.

It is possible to propagate and isolate quantities of retroviral vectors (e.g. to prepare  
20 suitable titres of the retroviral vector) for subsequent transduction of, for example, a site of interest by using a combination of a packaging or helper cell line and a recombinant vector.

In some instances, propagation and isolation may entail isolation of the retroviral *gag*,  
25 *pol* and *env* genes and their separate introduction into a host cell to produce a "packaging cell line". The packaging cell line produces the proteins required for packaging retroviral DNA but it cannot bring about encapsidation due to the lack of a *psi* region. However, when a recombinant vector carrying a NOI and a *psi* region is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant  
30 vector to produce the recombinant virus stock. This can be used to transduce cells to introduce the NOI into the genome of the cells. The recombinant virus whose genome

lacks all genes required to make viral proteins can transduce only once and cannot propagate. These viral vectors which are only capable of a single round of transduction of target cells are known as replication defective vectors. Hence, the NOI is introduced into the host/target cell genome without the generation of potentially harmful retrovirus.

5 A summary of the available packaging lines is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 449).

The design of retroviral packaging cell lines has evolved to address the problem of *inter alia* the spontaneous production of helper virus that was frequently encountered with

10 early designs. As recombination is greatly facilitated by homology, reducing or eliminating homology between the genomes of the vector and the helper has reduced the problem of helper virus production. More recently, packaging cells have been developed in which the *gag*, *pol* and *env* viral coding regions are carried on separate expression plasmids that are independently transfected into a packaging cell line so that

15 three recombinant events are required for wild type viral production. This reduces the potential for production of a replication-competent virus. This strategy is sometimes referred to as the three plasmid transfection method (Soneoka *et al* 1995 Nucl. Acids Res. 23: 628-633).

20 Transient transfection can also be used to measure vector production when vectors are being developed. In this regard, transient transfection avoids the longer time required to generate stable vector-producing cell lines and is used if the vector or retroviral packaging components are toxic to cells. Components typically used to generate retroviral vectors include a plasmid encoding the Gag/Pol proteins, a plasmid encoding

25 the Env protein and a plasmid containing a NOI. Vector production involves transient transfection of one or more of these components into cells containing the other required components. If the vector encodes toxic genes or genes that interfere with the replication of the host cell, such as inhibitors of the cell cycle or genes that induce apoptosis, it may be difficult to generate stable vector-producing cell lines, but transient

30 transfection can be used to produce the vector before the cells die. Also, cell lines have been developed using transient infection that produce vector titre levels that are

comparable to the levels obtained from stable vector-producing cell lines (Pear *et al* 1993, Proc Natl Acad Sci 90:8392-8396).

5 In view of the toxicity of some HIV proteins - which can make it difficult to generate stable HIV-based packaging cells - HIV vectors are usually made by transient transfection of vector and helper virus. Some workers have even replaced the HIV Env protein with that of vesicular stomatis virus (VSV). Insertion of the Env protein of VSV facilitates vector concentration as HIV/VSV-G vectors with titres of  $5 \times 10^5$  ( $10^8$  after  
10 concentration) have been generated by transient transfection (Naldini *et al* 1996 Science 272: 263-267). Thus, transient transfection of HIV vectors may provide a useful strategy for the generation of high titre vectors (Yee *et al* 1994 PNAS. 91: 9564-9568).

With regard to vector titre, the practical uses of retroviral vectors have been limited largely by the titres of transducing particles which can be attained in *in vitro* culture  
15 (typically not more than  $10^8$  particles/ml) and the sensitivity of many enveloped viruses to traditional biochemical and physicochemical techniques for concentrating and purifying viruses.

By way of example, several methods for concentration of retroviral vectors have been  
20 developed, including the use of centrifugation (Fekete and Cepko 1993 Mol Cell Biol 13: 2604-2613), hollow fibre filtration (Paul *et al* 1993 Hum Gene Ther 4: 609-615) and tangential flow filtration (Kotani *et al* 1994 Hum Gene Ther 5: 19-28). Although a 20-fold increase in viral titre can be achieved, the relative fragility of retroviral Env protein limits the ability to concentrate retroviral vectors and concentrating the virus usually  
25 results in a poor recovery of infectious virions. While this problem can be overcome by substitution of the retroviral Env protein with the more stable VSV-G protein, as described above, which allows for more effective vector concentration with better yields, it suffers from the drawback that the VSV-G protein is quite toxic to cells.

Although helper-virus free vector titres of  $10^7$  cfu/ml are obtainable with currently  
30 available vectors, experiments can often be done with much lower-titre vector stocks. However, for practical reasons, high-titre virus is desirable, especially when a large

number of cells must be infected. In addition, high titres are a requirement for transduction of a large percentage of certain cell types. For example, the frequency of human hematopoietic progenitor cell infection is strongly dependent on vector titre, and useful frequencies of infection occur only with very high-titre stocks (Hock and Miller 1986 Nature 320: 275-277; Hogge and Humphries 1987 Blood 69: 611-617). In these cases, it is not sufficient simply to expose the cells to a larger volume of virus to compensate for a low virus titre. On the contrary, in some cases, the concentration of infectious vector virions may be critical to promote efficient transduction.

Workers are trying to create high titre vectors for use in gene delivery. By way of example, a comparison of different vector designs has proved useful in helping to define the essential elements required for high-titre viral production. Early work on different retroviral vector design showed that almost all of the internal protein-encoding regions of MLVs could be deleted without abolishing the infectivity of the vector (Miller *et al* 1983 Proc Natl Acad Sci 80: 4709-4713). These early vectors retained only a small portion of the 3' end of the *env*-coding region. Subsequent work has shown that all of the *env*-gene-coding sequences can be removed without further reduction in vector titre (Miller and Rosman 1989 Biotechnology 7: 980-990; Morgenstern and Land 1990 Nucleic Acids Res 18: 3587-3596). Only the viral LTRs and short regions adjoining the LTRs, including the segments needed for plus- and minus-strand DNA priming and a region required for selective packaging of viral RNA into virions (the *psi* site; Mann *et al* 1983 Cell 33: 153-159) were deemed necessary for vector transmission. Nevertheless, viral titres obtained with these early vectors were still about tenfold lower than the parental helper virus titre.

Additional experiments indicated that retention of sequences at the 5' end of the *gag* gene significantly raised viral vector titres and that this was due to an increase in the packaging efficiency of viral RNA into virions (Armentano *et al* 1987 J Virol 61: 1647-1650; Bender *et al* 1987 J Virol 61: 1639-1646; Adam and Miller 1988 J Virol 62: 3802-3806). This effect was not due to viral protein synthesis from the *gag* region of the vector because disruption of the *gag* reading frame or mutating the *gag* codon to a

stop codon had no effect on vector titre (Bender *et al* 1987 *ibid*). These experiments demonstrated that the sequences required for efficient packaging of genomic RNA in MLV were larger than the *psi* signal previously defined by deletion analysis (Mann *et al* 1983 *ibid*). In order to obtain high titres ( $10^6$  to  $> 10^7$ ), it was shown to be important  
5 that this larger signal, called *psi* plus, be included in retroviral vectors. It has now been demonstrated that this signal spans from upstream of the splice donor to downstream of the *gag* start codon (Bender *et al* 1987 *ibid*). Because of this position, in spliced *env* expressing transcripts this signal is deleted. This ensures that only full length transcripts containing all three essential genes for viral life cycle are packaged.

10 In addition to manipulating the retroviral vector with a view to increasing vector titre, retroviral vectors have also been designed to induce the production of a specific NOI (usually a marker protein) in transduced cells. As already mentioned, the most common retroviral vector design involves the replacement of retroviral sequences with one or  
15 more NOIs to create replication-defective vectors. The simplest approach has been to use the promoter in the retroviral 5' LTR to control the expresssion of a cDNA encoding an NOI or to alter the enhancer/promoter of the LTR to provide tissue-specific expression or inducibility. Alternatively, a single coding region has been expressed by using an internal promoter which permits more flexibility in promoter selection.

20 These strategies for expression of a gene of interest have been most easily implemented when the NOI is a selectable marker, as in the case of hypoxanthine-guanine phosphoribosyl transferase (*hprt*) (Miller *et al* 1983 Proc Natl Acad Sci 80: 4709-4713) which facilitates the selection of vector transduced cells. If the vector contains an NOI  
25 that is not a selectable marker, the vector can be introduced into packaging cells by co-transfection with a selectable marker present on a separate plasmid. This strategy has an appealing advantage for gene therapy in that a single protein is expressed in the ultimate target cells and possible toxicity or antigenicity of a selectable marker is avoided. However, when the inserted gene is not selectable, this approach has the disadvantage  
30 that it is more difficult to generate cells that produce a high titre vector stock. In addition it is usually more difficult to determine the titre of the vector.

The current methodologies used to design retroviral vectors that express two or more proteins have relied on three general strategies. These include: (i) the expression of different proteins from alternatively spliced mRNAs transcribed from one promoter; (ii) the use of the promoter in the 5' LTR and internal promoters to drive transcription of different cDNAs and (iii) the use of internal ribosomal entry site (IRES) elements to allow translation of multiple coding regions from either a single mRNA or from fusion proteins that can then be expressed from an open reading frame.

Vectors containing internal promoters have been widely used to express multiple genes. An internal promoter makes it possible to exploit the promoter/enhancer combinations other than the viral LTR for driving gene expression. Multiple internal promoters can be included in a retroviral vector and it has proved possible to express at least three different cDNAs each from its own promoter (Overell *et al* 1988 Mol Cell Biol 8: 1803-1808).

While there now exist many such modified retroviral vectors which may be used for the expression of NOIs in a variety of mammalian cells, most of these retroviral vectors are derived from simple retroviruses such as murine oncoretroviruses that are incapable of transducing non-dividing cells.

By way of example, a widely used vector that employs alternative splicing to express genes from the viral LTR SV(X) (Cepko *et al* 1984 Cell 37: 1053-1062) contains the neomycin phosphotransferase gene as a selectable marker. The model for this type of vector is the parental virus, MO-MLV, in which the Gag and Gag-Pol proteins are translated from the full-length viral mRNA and the Env protein is made from the spliced mRNA. One of the proteins encoded by the vector is translated from the full-length RNA whereas splicing that links the splice donor near the 5'LTR to a splice acceptor just upstream of the second gene produces an RNA from which the second gene product can be translated. One drawback of this strategy is that foreign sequences are inserted into the intron of the spliced gene. This can affect the ratio of spliced to unspliced RNAs or provide alternative splice acceptors that interfere with production of the spliced RNA

encoding the second gene product (Korman *et al* 1987 Proc Natl Acad Sci 84: 2150-2154). Because these effects are unpredictable, they can affect the production of the encoded genes.

Other modified retroviral vectors can be divided into two classes with regards to splicing capabilities.

The first class of modified retroviral vector, typified by the pBABE vectors (Morgenstern *et al* 1990 Nucleic Acid Research 18: 3587-3596), contain mutations within the splice donor (GT to GC) that inhibit splicing of viral transcripts. Such splicing inhibition is beneficial for two reasons: Firstly, it ensures all viral transcripts contain a packaging signal and thus all can be packaged in the producer cell. Secondly, it prevents potential aberrant splicing between viral splice donors and possible cryptic splice acceptors of inserted genes.

The second class of modified retroviral vector, typified by both N2 (Miller *et al* 1989 Biotechniques 7: 980-990) and the more recent MFG (Dranoff *et al* 1993 Proc Natl Acad Sci 19: 3979-3986), contain functional introns. Both of these vectors use the normal splice donor found within the packaging signal. However, their respective splice acceptors (SAs) differ. For N2, the SA is found within the "extended" packaging signal (Bender *et al* 1987 *ibid*). For MFG, the natural SA (found within *pol*, see Figure 1 thereof) is used. For both these vectors, it has been demonstrated that splicing greatly enhances gene expression in transduced cells (Miller *et al* 1989 *ibid*; Krall *et al* 1996 Gene Therapy 3: 37-48). Such observations support previous findings that, in general, splicing can enhance mRNA translation (Lee *et al* 1981 Nature 294: 228-232; Lewis *et al* 1986 Mol Cell Biol 6: 1998-2010; Chapman *et al* 1991 Nucleic Acids Res 19: 3979-3986). One likely reason for this is that the same machinery involved in transcript splicing may also aid in transcript export from the nucleus.

Unlike the modified retroviral vectors described above, there has been very little work on alternative splicing in the retroviral lentiviral systems which are capable of infecting non-dividing cells (Naldini *et al* 1996 Science 272: 263-267). To date the only

published lentiviral vectors are those derived from HIV-1 (Kim *et al* 1997 J Virol 72: 811-816) and FIV (Poeschla *et al* 1998 Nat Med 4: 354-357). These vectors still contain virally derived splice donor and acceptor sequences (Naldini *et al* 1996 *ibid*).

- 5 Some alternative approaches to developing high titre vectors for gene delivery have included the use of: (i) defective viral vectors such as adenoviruses, adeno-associated virus (AAV), herpes viruses, and pox viruses and (ii) modified retroviral vector designs.

10 The adenovirus is a double-stranded, linear DNA virus that does not go through an RNA intermediate. There are over 50 different human serotypes of adenovirus divided into 6 subgroups based on the genetic sequence homology. The natural target of adenovirus is the respiratory and gastrointestinal epithelia, generally giving rise to only mild symptoms. Serotypes 2 and 5 (with 95% sequence homology) are most commonly used in adenoviral vector systems and are normally associated with upper respiratory tract infections in the  
15 young.

Adenoviruses are nonenveloped, regular icosahedrons. A typical adenovirus comprises a 140nm encapsidated DNA virus. The icosahedral symmetry of the virus is composed of 152 capsomeres: 240 hexons and 12 pentons. The core of the particle contains the 36kb  
20 linear duplex DNA which is covalently associated at the 5' ends with the Terminal Protein (TP) which acts as a primer for DNA replication. The DNA has inverted terminal repeats (ITR) and the length of these varies with the serotype.

Entry of adenovirus into cells involves a series of distinct events. Attachment of the virus  
25 to the cell occurs via an interaction between the viral fibre (37nm) and the fibre receptors on the cell. This receptor has recently been identified for Ad2/5 serotypes and designated as CAR (Coxsackie and Adeno Receptor, Tomko *et al* (1997 Proc Natl Acad Sci 94: 3352-2258). Internalisation of the virus into the endosome via the cellular  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins is mediated by and viral RGD sequence in the penton-base capsid protein  
30 (Wickham *et al.*, 1993 Cell 73: 309-319). Following internalisation, the endosome is disrupted by a process known as endosomolysis, an event which is believed to be



preferentially promoted by the cellular  $\alpha v \beta 5$  integrin (Wickham *et al.*, 1994 J Cell Biol 127: 257-264). In addition, there is recent evidence that the Ad5 fibre knob binds with high affinity to the MHC class 1  $\alpha 2$  domain at the surface of certain cell types including human epithelial and B lymphoblast cells (Hong *et al.*, 1997 EMBO 16: 2294-2306).

5

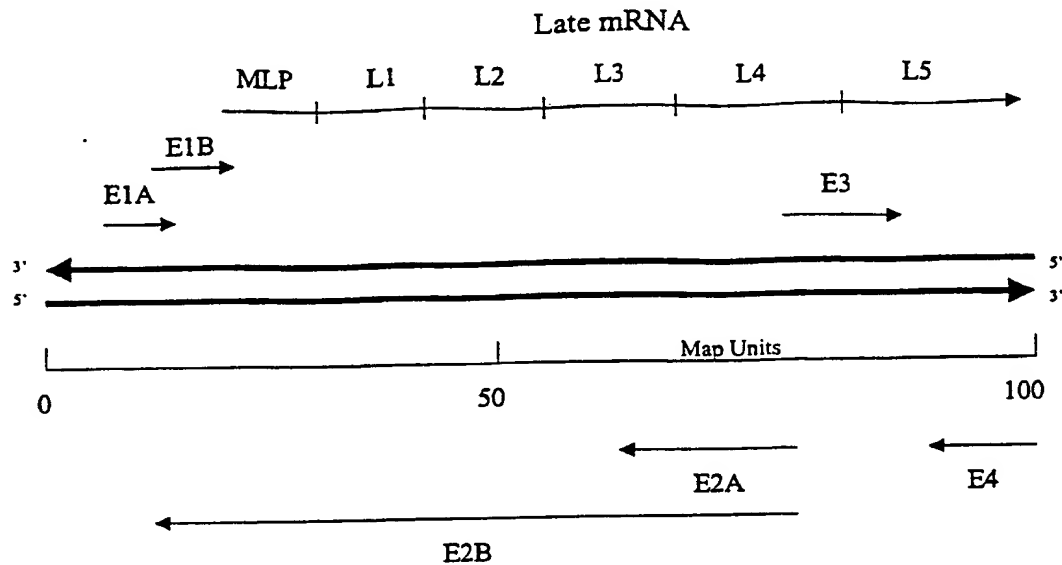
Subsequently the virus is translocated to the nucleus where activation of the early regions occurs and is shortly followed by DNA replication and activation of the late regions. Transcription, replication and packaging of the adenoviral DNA requires both host and viral functional protein machinery.

10

Viral gene expression can be divided into early (E) and late (L) phases. The late phase is defined by the onset of viral DNA replication. Adenovirus structural proteins are generally synthesised during the late phase. Following adenovirus infection, host cellular mRNA and protein synthesis is inhibited in cells infected with most serotypes. The adenovirus  
15 lytic cycle with adenovirus 2 and adenovirus 5 is very efficient and results in approximately 10, 000 virions per infected cell along with the synthesis of excess viral protein and DNA that is not incorporated into the virion. Early adenovirus transcription is a complicated sequence of interrelated biochemical events but it entails essentially the synthesis of viral RNAs prior to the onset of DNA replication.

20

The Schematic diagram below is of the adenovirus genome showing the relative direction and position of early and late gene transcription:



The organisation of the adenovirus genome is similar in all of the adenovirus groups and specific functions are generally positioned at identical locations for each serotype studied.

5 Early cytoplasmic messenger RNAs are complementary to four defined, noncontiguous regions on the viral DNA. These regions are designated E1-E4. The early transcripts have been classified into an array of intermediate early (E1a), delayed early (E1b, E2a, E2b, E3 and E4), and intermediate regions.

10 The early genes are expressed about 6-8 hours after infection and are driven from 7 promoters in gene blocks E1-4.

The E1a region is involved in transcriptional transactivation of viral and cellular genes as well as transcriptional repression of other sequences. The E1a gene exerts an important control function on all of the other early adenovirus messenger RNAs. In normal tissues, in order to transcribe regions E1b, E2a, E2b, E3 or E4 efficiently, active E1a product is required. However, the E1a function may be bypassed. Cells may be manipulated to provide E1a-like functions or may naturally contain such functions. The virus may also be manipulated to bypass the E1a function. The viral packaging signal overlaps with the E1a enhancer (194-358 nt).

15

20

The E1b region influences viral and cellular metabolism and host protein shut-off. It also includes the gene encoding the pIX protein (3525-4088 nt) which is required for packaging of the full length viral DNA and is important for the thermostability of the virus. The E1b region is required for the normal progression of viral events late in infection. The E1b product acts in the host nucleus. Mutants generated within the E1b sequences exhibit diminished late viral mRNA accumulation as well as impairment in the inhibition of host cellular transport normally observed late in adenovirus infection. E1b is required for altering functions of the host cell such that processing and transport are shifted in favour of viral late gene products. These products then result in viral packaging and release of virions. E1b produces a 19 kD protein that prevents apoptosis. E1b also produces a 55 kD protein that binds to p53. For a review on adenoviruses and their replication, see WO 96/17053.

The E2 region is essential as it encodes the 72 kDa DNA binding protein, DNA polymerase and the 80 kDa precursor of the 55 kDa Terminal Protein (TP) needed for protein priming to initiate DNA synthesis.

A 19 kDa protein (gp19K) is encoded within the E3 region and has been implicated in modulating the host immune response to the virus. Expression of this protein is upregulated in response to TNF alpha during the first phase of the infection and this then binds and prevents migration of the MHC class I antigens to the epithelial surface, thereby dampening the recognition of the adenoviral infected cells by the cytotoxic T lymphocytes. The E3 region is dispensable in *in vitro* studies and can be removed by deletion of a 1.9 kb *XbaI* fragment.

The E4 region is concerned with decreasing the host protein synthesis and increasing the DNA replication of the virus.

There are 5 families of late genes and all are initiated from the major late promoter. The expression of the late genes includes a very complex post-transcriptional control

mechanism involving RNA splicing. The fibre protein is encoded within the L5 region. The adenoviral genome is flanked by the inverted terminal repeat which in Ad5 is 103 bp and is essential for DNA replication. 30-40 hours post infection viral production is complete.

5

Adenoviruses may be converted for use as vectors for gene transfer by deleting the E1 gene, which is important for the induction of the E2, E3 and E4 promoters. The E1-replication defective virus may be propagated in a cell line that provides the E1 polypeptides in trans, such as the human embryonic kidney cell line 293. A therapeutic  
10 gene or genes can be inserted by recombination in place of the E1 gene. Expression of the gene is driven from either the E1 promoter or a heterologous promoter.

15

Even more attenuated adenoviral vectors have been developed by deleting some or all of the E4 open reading frames (ORFs). However, certain second generation vectors appear  
not to give longer-term gene expression, even though the DNA seems to be maintained. Thus, it appears that the function of one or more of the E4 ORFs may be to enhance gene expression from at least certain viral promoters carried by the virus.

20

An alternative approach to making a more defective virus has been to "gut" the virus  
completely maintaining only the terminal repeats required for viral replication. The "gutted" or "gutless" viruses can be grown to high titres with a first generation helper virus in the 293 cell line but it has been difficult to separate the "gutted" vector from the helper virus.

25

30

Replication-competent adenoviruses can also be used for gene therapy. For example, the E1A gene can be inserted into a first generation virus under the regulation of a tumour-specific promoter. In theory, following injection of the virus into a tumour, it could replicated specifically in the tumour but not in the surrounding normal cells. This type of vector could be used either to kill tumour cells directly by lysis or to deliver a "suicide  
gene" such as the herpes-simplex-virus thymidine-kinase gene (HSV *tk*) which can kill  
infected and bystander cells following treatment with ganciclovir. Alternatively, an

adenovirus defective only for E1b has been used specifically for antitumour treatment in phase-1 clinical trials. The polypeptides encoded by E1b are able to block p53-mediated apoptosis, preventing the cell from killing itself in response to viral infection. Thus, in normal nontumour cells, in the absence of E1b, the virus is unable to block apoptosis and is thus unable to produce infectious virus and spread. In tumour cells deficient in p53, the E1b defective virus can grow and spread to adjacent p53-defective tumour cells but not to normal cells. Again, this type of vector could also be used to deliver a therapeutic gene such as HSV *tk*.

- 10 The adenovirus provides advantages as a vector for gene delivery over other gene therapy vector systems for the following reasons:

It is a double stranded DNA nonenveloped virus that is capable of *in vivo* and *in vitro* transduction of a broad range of cell types of human and non-human origin. These cells include respiratory airway epithelial cells, hepatocytes, muscle cells, cardiac myocytes, synoviocytes, primary mammary epithelial cells and post-mitotically terminally differentiated cells such as neurons (with perhaps the important exception of some lymphoid cells including monocytes).

Adenoviral vectors are also capable of transducing non dividing cells. This is very important for diseases, such as cystic fibrosis, in which the affected cells in the lung epithelium, have a slow turnover rate. In fact, several trials are underway utilising adenovirus-mediated transfer of cystic fibrosis transporter (CFTR) into the lungs of afflicted adult cystic fibrosis patients.

25 Adenoviruses have been used as vectors for gene therapy and for expression of heterologous genes. The large (36 kilobase) genome can accommodate up to 8kb of foreign insert DNA and is able to replicate efficiently in complementing cell lines to produce very high titres of up to  $10^{12}$ . Adenovirus is thus one of the best systems to study the expression of genes in primary non-replicative cells.

30

The expression of viral or foreign genes from the adenovirus genome does not require a

replicating cell. Adenoviral vectors enter cells by receptor mediated endocytosis. Once inside the cell, adenovirus vectors rarely integrate into the host chromosome. Instead, it functions episomally (independently from the host genome) as a linear genome in the host nucleus. Hence the use of recombinant adenovirus alleviates the problems associated with random integration into the host genome.

There is no association of human malignancy with adenovirus infection. Attenuated adenoviral strains have been developed and have been used in humans as live vaccines.

However, current adenoviral vectors suffer from some major limitations for *in vivo* therapeutic use. These include: (i) transient gene expression- the adenoviral vector generally remains episomal and does not replicate so that it is not passed onto subsequent progeny (ii) because of its inability to replicate, target cell proliferation can lead to dilution of the vector (iii) an immunological response raised against the adenoviral proteins so that cells expressing adenoviral proteins, even at a low level, are destroyed and (iv) an inability to achieve an effective therapeutic index since *in vivo* delivery leads to an uptake of the vector and expression of the delivered genes in only a proportion of target cells.

If the features of adenoviruses can be combined with the genetic stability of retro/lentiviruses then essentially the adenovirus can be used to transduce target cells to become transient retroviral producer cells that can stably infect neighbouring cells.

The present invention seeks to provide a novel retroviral vector.

In particular, the present invention seeks to provide a novel retroviral vector capable of providing efficient expression of a NOI - or even a plurality of NOIs - at one or more desired target sites.

The present invention also seeks to provide a novel system for preparing high titres of vector virion which incorporates safety features for *in vivo* use and which is capable of

providing efficient expression of a NOI - or even a plurality of NOIs - at one or more desired target sites.

According to a first aspect of the present invention, there is provided a retroviral vector  
5 comprising a functional splice donor site and a functional splice acceptor site; wherein  
the functional splice donor site and the functional splice acceptor site flank a first  
nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is  
upstream of the functional splice acceptor site; wherein the retroviral vector is derived  
10 from a retroviral pro-vector; wherein the retroviral pro-vector comprises a first  
nucleotide sequence (NS) capable of yielding the functional splice donor site and a  
second NS capable of yielding the functional splice acceptor site; wherein the first NS is  
downstream of the second NS; such that the retroviral vector is formed as a result of  
reverse transcription of the retroviral pro-vector.

15 According to a second aspect of the present invention, there is provided a retroviral  
vector wherein the retroviral pro-vector comprises a retroviral packaging signal; and  
wherein the second NS is located downstream of the retroviral packaging signal such that  
splicing is preventable at a primary target site.

20 According to a third aspect of the present invention, there is provided a retroviral vector  
wherein the second NS is placed downstream of the first NOI such that the first NOI is  
capable of being expressed at a primary target site.

According to a fourth aspect of the present invention, there is provided a retroviral  
25 vector wherein the second NS is placed upstream of a multiple cloning site such that one  
or more additional NOIs may be inserted.

According to a fifth aspect of the present invention, there is provided a retroviral vector  
wherein the second NS is a nucleotide sequence coding for an immunological molecule  
30 or a part thereof.

According to a sixth aspect of the present invention, there is provided a retroviral vector wherein the immunological molecule is an immunoglobulin.

5 According to a seventh aspect of the present invention, there is provided a retroviral vector wherein the second NS is a nucleotide sequence coding for an immunoglobulin heavy chain variable region.

According to a eighth aspect of the present invention, there is provided a retroviral vector wherein the vector additionally comprises a functional intron.

10

According to a ninth aspect of the present invention, there is provided a retroviral vector wherein the functional intron is positioned so that it is capable of restricting expression of at least one of the NOIs in a desired target site.

15 According to a tenth aspect of the present invention, there is provided a retroviral vector wherein the target site is a cell.

20 According to a eleventh aspect of the present invention, there is provided a retroviral vector wherein the vector or pro-vector is derivable from a murine oncoretrovirus or a lentivirus.

According to a twelfth aspect of the present invention, there is provided a retroviral vector wherein the vector is derivable from MMLV, MSV, MMTV, HIV-1 or ELAV.

25 According to a thirteenth aspect of the present invention, there is provided a retroviral vector wherein the retroviral vector is an integrated provirus.

According to a fourteenth aspect of the present invention, there is provided a retroviral particle obtainable from a retroviral vector.

30



According to a fifteenth aspect of the present invention, there is provided a cell transfected or transduced with a retroviral vector.

5 According to a sixteenth aspect of the present invention there is provided a retroviral vector or a viral particle or a cell for use in medicine.

According to a seventeenth aspect of the present invention there is provided a retroviral vector or a viral particle or a cell for the manufacture of a pharmaceutical composition to deliver one or more NOIs to a target site in need of same.

10

According to an eighteenth aspect of the present invention there is provided a method comprising transfecting or transducing a cell with a retroviral vector or a viral particle or by use of a cell.

15

According to a nineteenth aspect of the present invention there is provided a delivery system for a retroviral vector or a viral particle or a cell wherein the delivery system comprises one or more non-retroviral expression vector(s), adenoviruse(s), or plasmid(s) or combinations thereof for delivery of an NOI or a plurality of NOIs to a first target cell and a retroviral vector for delivery of an NOI or a plurality of NOIs to a second

20

target cell.

According to a twentieth aspect of the present invention there is provided a retroviral pro-vector.

25 According to a twenty first aspect of the present invention there is provided the use of a functional intron to restrict expression of one or more NOIs within a desired target cell.

According to a twenty second aspect of the present invention there is provided the use of a reverse transcriptase to deliver a first NS from the 3' end of a retroviral pro-vector to

30

the 5' end of a retroviral vector.

According to a twenty third aspect of the present invention there is provided a hybrid viral vector system for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which  
5 secondary vector is capable of transducing a secondary target cell.

According to a twenty fourth aspect of the present invention there is provided a hybrid viral vector system wherein the primary vector is obtainable from or is based on a adenoviral vector and/or the secondary viral vector is obtainable from or is based on a  
10 retroviral vector preferably a lentiviral vector.

According to a twenty fifth aspect of the present invention there is provided a hybrid viral vector system wherein the lentiviral vector comprises or is capable of delivering a split-intron configuration.

15 According to a twenty sixth aspect of the present invention there is provided a lentiviral vector system wherein the lentiviral vector comprises or is capable of delivering a split-intron configuration.

20 According to a twenty seventh aspect of the present invention there is provided an adenoviral vector system wherein the adenoviral vector comprises or is capable of delivering a split-intron configuration.

According to a twenty eighth aspect of the present invention there is provided vectors or  
25 plasmids based on or obtained from any one or more of the entities presented as pE1sp1A, pCI-Neo, pE1RevE, pE1HORSE3.1, pE1PEGASUS4, pCI-Rab, pE1Rab.

According to a twenty ninth aspect of the present invention there is provided a retroviral vector capable of differential expression of NOIs in target cells.

30

Another aspect of the present invention includes a hybrid viral vector system for *in vivo* gene delivery, which system comprises a primary viral vector which encodes a secondary viral vector, the primary vector capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell, wherein the primary vector is obtainable from or is based on an adenoviral vector and the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector.

Another aspect of the present invention includes a hybrid viral vector system for *in vivo* gene delivery, which system comprises a primary viral vector which encodes a secondary viral vector, the primary vector capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell, wherein the primary vector is obtainable from or is based on an adenoviral vector and the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector; wherein the viral vector system comprises a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector; wherein the retroviral pro-vector comprises a first nucleotide sequence ("NS") capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS; such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.

Preferably the retroviral pro-vector comprises a third NS that is upstream of the second nucleotide sequence; wherein the third NS is capable of yielding a non-functional splice donor site.

Preferably the retroviral vector further comprises a second NOI; wherein the second NOI is downstream of the functional splice acceptor site.

Preferably the retroviral pro-vector comprises the second NOI; wherein the second NOI is downstream of the second nucleotide sequence.

- 5 Preferably the second NOI, or the expression product thereof, is or comprises a therapeutic agent or a diagnostic agent.

Preferably the first NOI, or the expression product thereof, is or comprises any one or more of an agent conferring selectability (e.g. a marker element), a viral essential  
10 element, or a part thereof, or combinations thereof.

Preferably the first NS is at or near to the 3' end of a retroviral pro-vector; preferably wherein the 3' end comprises a U3 region and an R region; and preferably wherein the first NS is located between the U3 region and the R region.

15

Preferably the U3 region and/or the first NS of the retroviral pro-vector comprises an NS that is a third NOI; wherein the NOI is any one or more of a transcriptional control element, a coding sequence or a part thereof.

- 20 Preferably the first NS is obtainable from a virus.

Preferably the first NS is an intron or a part thereof.

Preferably the intron is obtainable from the small t-intron of SV40 virus.

25

Preferably the vector components are regulated. In one preferred aspect of the invention,  
the vector components are regulated by hypoxia.

- 30 In another preferred aspect of the invention, the vector components are regulated by tetracycline on/off system.

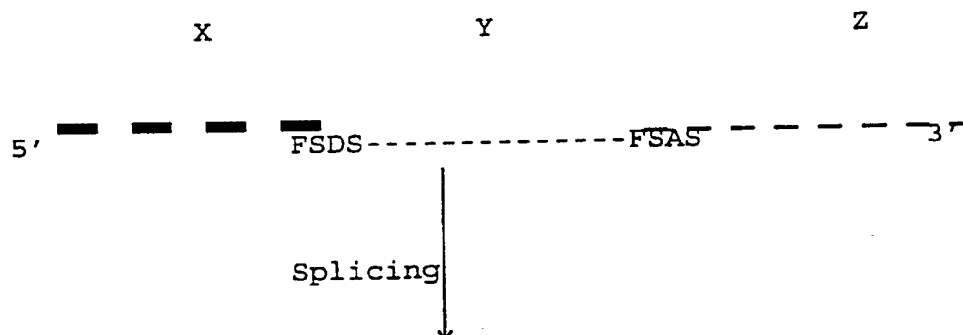
Thus, the present invention provides a delivery system which utilises a retroviral vector.

The retroviral vector of the delivery system of the present invention comprises a functional splice donor site ("FSDS") and a functional splice acceptor site ("FSAS") which flank a first NOI. The retroviral vector is formed as a result of reverse transcription of a retroviral pro-vector which may comprise a plurality of NOIs.

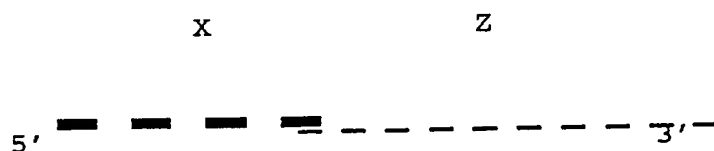
When the FSDS is positioned upstream of the FSAS, any intervening sequence(s) are capable of being spliced. Typically, splicing removes intervening or "intronic" RNA sequences and the remaining "exonic" sequences are ligated to provide continuous sequences for translation.

The splicing process can be pictorially represented as:

#### Unspliced Form



#### Spliced Form



In this pictorial representation, Y represents the intervening sequence that is removed as a result of splicing.

The natural splicing configuration for retroviral vectors is shown in Figure 27a. The splicing configuration of known vectors is shown in Figure 27b. The Splicing configuration according to the present invention is shown in Figure 27c.

- 5 In accordance with the present invention, if the FSDS is downstream of the FSAS, then splicing cannot occur.

Likewise, if the FSDS is a non-functional splice donor site (NFSDS) and/or the FSAS is a non-functional acceptor site (NFAS), then splicing cannot occur.

10

An example of a NFSDS is a mutated FSDS such that the FSDS can no longer be recognised by the splicing mechanism.

- 15 In accordance with the present invention, each NS can be any suitable nucleotide sequence. For example, each sequence can be independently DNA or RNA - which may be synthetically prepared or may be prepared by use of recombinant DNA techniques or may be isolated from natural sources or may be combinations thereof. The sequence may be a sense sequence or an antisense sequence. There may be a plurality of sequences, which may be directly or indirectly joined to each other, or combinations thereof.
- 20

- In accordance with the present invention, each NOI can be any suitable nucleotide sequence. For example, each sequence can be independently DNA or RNA - which may be synthetically prepared or may be prepared by use of recombinant DNA techniques or may be isolated from natural sources or may be combinations thereof. The sequence may be a sense sequence or an antisense sequence. There may be a plurality of sequences, which may be directly or indirectly joined to each other, or combinations thereof.
- 25

- 30 The first NOI may include any one or more of the following selectable markers which have been used successfully in retroviral vectors: the bacterial neomycin and hygromycin

phosphotransferase genes which confer resistance to G418 and hygromycin respectively (Palmer *et al* 1987 Proc Natl Acad Sci 84: 1055-1059; Yang *et al* 1987 Mol Cell Biol 7: 3923-3928); a mutant mouse dihydrofolate reductase gene (*dhfr*) which confers resistance to methotrexate (Miller *et al* 1985 Mol Cell Biol 5: 431-437); the bacterial *gpt* gene  
5 which allows cells to grow in medium containing mycophenolic acid, xanthine and aminopterin (Mann *et al* 1983 Cell 33: 153-159); the bacterial *hisD* gene which allows cells to grow in medium without histidine but containing histidinol (Danos and Mulligan 1988 Proc Natl Acad Sci 85: 6460-6464); the multidrug resistance gene (*mdr*) which confers resistance to a variety of drugs (Guild *et al* 1988 Proc Natl Acad Sci 85: 1595-  
10 1599; Pastan *et al* 1988 Proc Natl Acad Sci 85: 4486-4490) and the bacterial genes which confer resistance to puromycin or phleomycin (Morgenstern and Land 1990 Nucleic Acid Res 18: 3587-3596).

All of these markers are dominant selectable markers and allow chemical selection of  
15 most cells expressing these genes.  $\beta$ -galactosidase can also be considered a dominant marker; cells expressing  $\beta$ -galactosidase can be selected by using the fluorescence-activated cell sorter. In fact, any cell surface protein can provide a selectable marker for cells not already making the protein. Cells expressing the protein can be selected by using the fluorescent antibody to the protein and a cell sorter. Other selectable markers  
20 that have been included in vectors include the *hprt* and HSV thymidine kinase which allows cells to grow in medium containing hypoxanthine, aminopterin and thymidine.

The first NOI could contain non-coding sequences, for example the retroviral packaging site or non-sense sequences that render the second NOI non-functional in the provector  
25 but when they are removed by the splicing the vector the second NOI is revealed for functional expression.

The first NOI may also encode a viral essential element such as *env* encoding the Env protein which can reduce the complexity of production systems. By way of example, in  
30 an adenoviral vector, this allows the retroviral vector genome and the envelope to be configured in a single adenoviral vector under the same promoter control thus providing

a simpler system and leaving more capacity in the adenoviral vector for additional sequences. In one aspect, those additional sequences could be the gag-pol cassette itself. Thus in one adenoviral vector one can produce a retroviral vector particle. Previous studies (Feng et al 1997 Nature Biotechnology 15: 866) have required the use of multiple  
5 adenoviral vectors.

If the retroviral component includes an *env* nucleotide sequence, then all or part of that sequence can be optionally replaced with all or part of another *env* nucleotide sequence such as, by way of example, the amphotropic Env protein designated 4070A or the  
10 influenza haemagglutinin (HA) or the vesicular stomatitis virus G (VSV-G) protein. Replacement of the *env* gene with a heterologous *env* gene is an example of a technique or strategy called pseudotyping. Pseudotyping is not a new phenomenon and examples may be found in WO-A-98/05759, WO-A-98/05754, WO-A-97/17457, WO-A-96/09400, WO-A-91/00047 and Mebatsion *et al* 1997 Cell 90, 841-847.

15 In one preferred aspect, the retroviral vector of the present invention has been pseudotyped. In this regard, pseudotyping can confer one or more advantages. For example, with the lentiviral vectors, the *env* gene product of the HIV based vectors would restrict these vectors to infecting only cells that express a protein called CD4.  
20 But if the *env* gene in these vectors has been substituted with *env* sequences from other RNA viruses, then they may have a broader infectious spectrum (Verma and Somia 1997 Nature 389:239-242). By way of example, workers have pseudotyped an HIV based vector with the glycoprotein from VSV (Verma and Somia 1997 *ibid*).

25 In another alternative, the Env protein may be a modified Env protein such as a mutant or engineered Env protein. Modifications may be made or selected to introduce targeting ability or to reduce toxicity or for another purpose (Valsecia-Wittman *et al* 1996 J Virol 70: 2056-64; Nilson *et al* 1996 Gene Therapy 3: 280-6; Fielding *et al* 1998 Blood 9: 1802 and references cited therein).



Suitable second NOI coding sequences include those that are of therapeutic and/or diagnostic application such as, but are not limited to: sequences encoding cytokines, chemokines, hormones, antibodies, engineered immunoglobulin-like molecules, a single chain antibody, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a tumour suppressor protein and growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives thereof (such as with an associated reporter group). When included, such coding sequences may be typically operatively linked to a suitable promoter, which may be a promoter driving expression of a ribozyme(s), or a different promoter or promoters.

The second NOI coding sequence may encode a fusion protein or a segment of a coding sequence

15

The retroviral vector of the present invention may be used to deliver a second NOI such as a pro-drug activating enzyme to a tumour site for the treatment of a cancer. In each case, a suitable pro-drug is used in the treatment of the individual (such as a patient) in combination with the appropriate pro-drug activating enzyme. An appropriate pro-drug is administered in conjunction with the vector. Examples of pro-drugs include: etoposide phosphate (with alkaline phosphatase, Senter *et al* 1988 Proc Natl Acad Sci 85: 4842-4846); 5-fluorocytosine (with cytosine deaminase, Mullen *et al* 1994 Cancer Res 54: 1503-1506); Doxorubicin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase, Kerr *et al* 1990 Cancer Immunol Immunother 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with  $\beta$ -lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli *et al* 1988 Proc Natl Acad Sci 85: 7572-7576); mustard pro-drugs with nitroreductase (Friedlos *et al* 1997 J Med Chem 40: 1270-1275) and Cyclophosphamide (with P450 Chen *et al* 1996 Cancer Res 56: 1331-1340).

30

The vector of the present invention may be delivered to a target site by a viral or a non-viral vector.

As it is well known in the art, a vector is a tool that allows or facilitates the transfer of an entity from one environment to another. By way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

Non-viral delivery systems include but are not limited to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a gene to a target mammalian cell.

Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), and combinations thereof.

Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, retroviral vector, lentiviral vector, baculoviral vector. Other examples of vectors include *ex vivo* delivery systems, which include but are not limited to DNA transfection methods such as electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection.

The vector delivery system of the present invention may consist of a primary vector manufactured *in vitro* which encodes the genes necessary to produce a secondary vector *in vivo*.

The primary viral vector or vectors may be a variety of different viral vectors, such as retroviral, adenoviral, herpes virus or pox virus vectors, or in the case of multiple primary viral vectors, they may be a mixture of vectors of different viral origin. In whichever case, the primary viral vectors are preferably defective in that they are incapable of independent replication. Thus, they are capable of entering a target cell and delivering the secondary vector sequences, but not of replicating so as to go on to infect further target cells.

In the case where the hybrid viral vector system comprises more than one primary vector to encode the secondary vector, both or all three primary vectors will be used to transfect or transduce a primary target cell population, usually simultaneously.

Preferably, there is a single primary viral vector which encodes all components of the secondary viral vector.

15

The preferred single or multiple primary viral vectors are adenoviral vectors.

Adenoviral vectors for use in the invention may be derived from a human adenovirus or an adenovirus which does not normally infect humans. Preferably the vectors are derived from adenovirus type 2 or adenovirus type 5 (Ad2 or Ad5) or a mouse adenovirus or an avian adenovirus such as CELO virus (Cotton *et al* 1993 J Virol 67:3777-3785). The vectors may be replication competent adenoviral vectors but are more preferably defective adenoviral vectors. Adenoviral vectors may be rendered defective by deletion of one or more components necessary for replication of the virus. Typically, each adenoviral vector contains at least a deletion in the E1 region. For production of infectious adenoviral vector particles, this deletion may be complemented by passage of the virus in a human embryo fibroblast cell line such as human 293 cell line, containing an integrated copy of the left portion of Ad5, including the E1 gene. The capacity for insertion of heterologous DNA into such vectors can be up to approximately 7kb. Thus such vectors are useful for construction of a system according

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to the invention comprising three separate recombinant vectors each containing one of the essential transcription units for construction of the retroviral secondary vector.

Alternative adenoviral vectors are known in the art which contain further deletions in other adenoviral genes and these vectors are also suitable for use in the invention. Several of these second generation adenoviral vectors show reduced immunogenicity (eg E1 + E2 deletions Gorziglia *et al* 1996 J Virol 70: 4173-4178; E1 + E4 deletions Yeh *et al* 1996 J Virol 70: 559-565). Extended deletions serve to provide additional cloning capacity for the introduction of multiple genes in the vector. For example a 25 kb deletion has been described (Lieber *et al* 1996 J Virol 70: 8944-8960) and a cloning vector deleted of all viral genes has been reported (Fisher *et al* 1996 Virology 217: 11-22) which permit the introduction of more than 35 kb of heterologous DNA. Such vectors may be used to generate an adenoviral primary vector according to the invention encoding two or three transcription units for construction of the retroviral secondary vector.

The secondary viral vector is preferably a retroviral vector. The secondary vector is produced by expression of essential genes for assembly and packaging of a defective viral vector particle, within the primary target cells. It is defective in that it is incapable of independent replication. Thus, once the secondary retroviral vector has transduced a secondary target cell, it is incapable of spreading by replication to any further target cells.

The term "retroviral vector" typically includes a retroviral nucleic acid which is capable of infection, but which is not capable, by itself, of replication. Thus it is replication defective. A retroviral vector typically comprises one or more NOI(s), preferably of non-retroviral origin, for delivery to target cells. A retroviral vector may also comprises a functional splice donor site (FSDS) and a functional splice acceptor site (FSAS) so that when the FSDS is upstream of the FSAS, any intervening sequence(s) are capable of being spliced. A retroviral vector may comprise further non-retroviral sequences, such as non-retroviral control sequences in the U3 region which may influence expression of

an NOI(s) once the retroviral vector is integrated as a provirus into a target cell. The retroviral vector need not contain elements from only a single retrovirus. Thus, in accordance with the present invention, it is possible to have elements derivable from two of more different retroviruses or other sources

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The term "retroviral pro-vector" typically includes a retroviral vector genome as described above but which comprises a first nucleotide sequence (NS) capable of yielding a functional splice donor site (FSDs) and a second NS capable of yielding a functional splice acceptor site (FSAS) such that the first NS is downstream of the second NS so that  
10 splicing associated with the first NS and the second NS cannot occur. Upon reverse transcription of the retroviral pro-vector, a retroviral vector is formed.

The term "retroviral vector particle" refers to the packaged retroviral vector, that is preferably capable of binding to and entering target cells. The components of the  
15 particle, as already discussed for the vector, may be modified with respect to the wild type retrovirus. For example, the Env proteins in the proteinaceous coat of the particle may be genetically modified in order to alter their targeting specificity or achieve some other desired function.

20 The retroviral vector of this aspect of the invention may be derivable from a murine oncoretrovirus such as MMLV, MSV or MMTV; or may be derivable from a lentivirus such as HIV-1, EIAV; or may be derivable from another retrovirus.

The retroviral vector of the invention can be modified to render the natural splice donor  
25 site of the retrovirus non-functional.

The term "modification" includes the silencing or removal of the natural splice donor. Vectors, such as MLV based vectors, which have the splice donor site removed are known in the art. An example of such a vector is pBABE (Morgenstern *et al* 1990 *ibid*).

30

The secondary vector may be produced from expression of essential genes for retroviral vector production encoded in the DNA of the primary vector. Such genes may include a *gag-pol* gene from a retrovirus, an *env* gene from an enveloped virus and a defective retroviral vector containing one or more therapeutic or diagnostic NOI(s). The defective retroviral vector contains in general terms sequences to enable reverse transcription, at least part of a 5' long terminal repeat (LTR), at least part of a 3'LTR and a packaging signal.

If it is desired to render the secondary vector replication defective, that secondary vector may be encoded by a plurality of transcription units, which may be located in a single or in two or more adenoviral or other primary vectors. Thus, there may be a transcription unit encoding the secondary vector genome, a transcription unit encoding *gag-pol* and a transcription unit encoding *env*. Alternatively, two or more of these may be combined. For example, nucleic acid sequences encoding *gag-pol* and *env*, or *env* and the genome, may be combined in a single transcription unit. Ways of achieving this are known in the art.

Transcription units as described herein are regions of nucleic acid containing coding sequences and the signals for achieving expression of those coding sequences independently of any other coding sequences. Thus, each transcription unit generally comprises at least a promoter, an enhancer and a polyadenylation signal.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.

The term "enhancer" includes a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

The promoter and enhancer of the transcription units encoding the secondary vector are preferably strongly active, or capable of being strongly induced, in the primary target

cells under conditions for production of the secondary viral vector. The promoter and/or enhancer may be constitutively efficient, or may be tissue or temporally restricted in their activity. Examples of suitable tissue restricted promoters/enhancers are those which are highly active in tumour cells such as a promoter/enhancer from a MUC1 gene, a CEA gene or a 5T4 antigen gene. Examples of temporally restricted promoters/enhancers are those which are responsive to ischaemia and/or hypoxia, such as hypoxia response elements or the promoter/enhancer of a grp78 or a grp94 gene. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

Other preferred additional components include entities enabling efficient expression of an NOI or a plurality of NOIs.

In one preferred aspect of the present invention, there is hypoxia or ischaemia regulatable expression of the secondary vector components. In this regard, hypoxia is a powerful regulator of gene expression in a wide range of different cell types and acts by the induction of the activity of hypoxia-inducible transcription factors such as hypoxia inducible factor-1 (HIF-1; Wang & Semenza 1993 Proc Natl Acad Sci 90:430), which bind to cognate DNA recognition sites, the hypoxia-responsive elements (HREs) on various gene promoters. Dachs *et al* (1997 Nature Med 5: 515) have used a multimeric form of the HRE from the mouse phosphoglycerate kinase-1 (PGK-1) gene (Firth *et al* 1994 Proc Natl Acad Sci 91:6496-6500) to control expression of both marker and therapeutic genes by human fibrosarcoma cells in response to hypoxia *in vitro* and within solid tumours *in vivo* (Dachs *et al* *ibid*). Alternatively, the fact that marked glucose deprivation is also present in ischaemic areas of tumours can be used to activate heterologous gene expression specifically in tumours. A truncated 632 base pair sequence of the grp 78 gene promoter, known to be activated specifically by glucose deprivation, has also been shown to be capable of driving high level expression of a reporter gene in murine tumours *in vivo* (Gazit *et al* 1995 Cancer Res 55:1660).

An alternative method of regulating the expression of such components is by using the tetracycline on/off system described by Gossen and Bujard (1992 Proc Natl Acad Sci 89: 5547) as described for the production of retroviral *gal*, *pol* and VSV-G proteins by Yoshida *et al* (1997 Biochem Biophys Res Comm 230: 426). Unusually this regulatory  
5 system is also used in the present invention to control the production of the pro-vector genome. This ensures that no vector components are expressed from the adenoviral vector in the absence of tetracycline.

Safety features which may be incorporated into the hybrid viral vector system are  
10 described below. One or more such features may be present.

The secondary vector is also advantageous for *in vivo* use in that incorporated into it are one or more features which eliminate the possibility of recombination to produce an infectious virus capable of independent replication. Such features were not included in  
15 previous published studies (Feng *et al* 1997 *ibid*). In particular, the construction of a retroviral vector from three components as described below was not described by Feng *et al* (*ibid*).

Firstly, sequence homology between the sequences encoding the components of the  
20 secondary vector may be avoided by deletion of regions of homology. Regions of homology allow genetic recombination to occur. In a particular embodiment, three transcription units are used to construct a secondary retroviral vector. The first transcription unit contains a retroviral *gag-pol* gene under the control of a non-retroviral promoter and enhancer. The second transcription unit contains a retroviral *env* gene  
25 under the control of a non-retroviral promoter and enhancer. The third transcription unit comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. In the native retroviral genome, the packaging signal is located such that part of the *gag* sequence is required for proper functioning. Normally when retroviral vector systems are constructed therefrom, the packaging signal, including part of the *gag*  
30 gene, remains in the vector genome. In the present case however, the defective retroviral genome contains a minimal packaging signal which does not contain sequences



homologous to *gag* sequences in the first transcription unit. Also, in retroviruses, for example Moloney Murine Leukaemia virus (MMLV), there is a small region of overlap between the 3' end of the *pol* coding sequence and the 5' end of *env*. The corresponding region of homology between the first and second transcription units may be removed by  
5 altering the sequence of either the 3' end of the *pol* coding sequence or the 5' end of *env* so as to change the codon usage but not the amino acid sequence of the encoded proteins.

Secondly, the possibility of replication competent secondary viral vectors may be avoided by pseudotyping the genome of one retrovirus with the Env protein of another  
10 retrovirus or another enveloped virus so that regions of homology between the *env* and *gag-pol* components are avoided.

In a particular embodiment the retroviral vector is constructed from the following three components: The first transcription unit contains a retroviral *gag-pol* gene under the control of a non-retroviral promoter and enhancer. The second transcription unit  
15 contains the *env* gene from the alternative enveloped virus, under the control of a non-retroviral promoter and enhancer. The third transcription unit comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. The defective retroviral genome contains a minimal packaging signal which does not contain  
20 sequences homologous to *gag* sequences in the first transcription unit.

Thirdly, the possibility of replication competent retroviruses can be eliminated by using two transcription units constructed in a particular way. The first transcription unit contains a *gag-pol* coding region under the control of a promoter-enhancer active in the  
25 primary target cell such as a hCMV promoter-enhancer or a tissue restricted promoter-enhancer. The second transcription unit encodes a retroviral genome RNA capable of being packaged into a retroviral particle. The second transcription unit contains retroviral sequences necessary for packaging, integration and reverse transcription and also contains sequences coding for an *env* protein of an enveloped virus and the coding  
30 sequence of one or more therapeutic genes.

In this example, the transcription of the *env* and an NOI coding sequences is devised such that the Env protein is preferentially produced in the primary target cell while the NOI expression product is or are preferentially produced in the secondary target cell.

5 A suitable intron splicing arrangement is described later on in Example 5 and illustrated in Figure 17 and Figure 27c. Here, a splice donor site is positioned downstream of a splice acceptor site in the retroviral genome sequence delivered by the primary vector to the primary target cell. Splicing will therefore be absent or infrequent in the primary target cell so the Env protein will preferentially be expressed. However, once the vector  
10 genome has gone through the process of reverse transcription and integration into the secondary target cell, a functional splice donor sequence will be located in the 5' LTR, upstream of a functional splice acceptor sequence. Splicing occurs to splice out the *env* sequence and transcripts of the NOI are produced.

15 In a second arrangement of this example, the expression of an NOI is restricted to the secondary target cell and prevented from being expressed in the primary target cell as follows: This arrangement is described later on in Example 6 and illustrated in Figure 18. There, a promoter-enhancer and a first fragment of an NOI containing the 5' end of the coding sequence and a natural or artificially derived or derivable splice donor  
20 sequence are inserted at the 3' end of the retroviral genome construct upstream of the R-region. A second fragment of the NOI which contains all the sequences required to complete the coding region is placed downstream of a natural or artificially derived or derivable splice acceptor sequence located downstream from the packaging signal in the retroviral genome construct. On reverse transcription and integration of the retroviral  
25 genome in the secondary target cell, the promoter 5' fragment of the NOI and the functional splice donor sequence are located upstream of the functional splice acceptor and the 3' end of the NOI. Transcription from the promoter and splicing then permit translation of the NOI in the secondary target cell.

In a preferred embodiment the hybrid viral vector system according to the invention comprises single or multiple adenoviral primary vectors which encodes or encode a retroviral secondary vector.

- 5 Preferred embodiments of the present invention described address one of the major problems associated with adenoviral and other viral vectors, namely that gene expression from such vectors is transient. The retroviral particles generated from the primary target cells can transduce secondary target cells and gene expression in the secondary target cells is stably maintained because of the integration of the retroviral vector genome into  
10 the host cell genome. The secondary target cells do not express significant amounts of viral protein antigens and so are less immunogenic than cells transduced with adenoviral vector.

- The use of a retroviral vector as the secondary vector is advantageous because it allows a  
15 degree of cellular discrimination, for instance by permitting the targeting of rapidly dividing cells. Furthermore, retroviral integration permits the stable expression of therapeutic genes in the target tissue, including stable expression in proliferating target cells.

- 20 The use of the novel retroviral vector design of the present invention is also advantageous in that gene expression can be limited to a primary or a secondary target site. In this way, single or multiple NOIs can be preferentially expressed at a secondary target site and poorly expressed or not expressed at a biologically significant level at a primary target site. As a result, the possible toxicity or antigenicity of an NOI may be  
25 avoided.

Preferably, the primary viral vector preferentially transduces a certain cell type or cell types.

More preferably, the primary vector is a targeted vector, that is it has a tissue tropism which is altered compared to the native virus, so that the vector is targeted to particular cells.

- 5 The term "targeted vector" is not necessarily linked to the term "target site" or target cell".

"Target site" refers to a site which a vector, whether native or targeted, is capable of transfecting or transducing.

10

"Primary target site" refers to a first site which a vector, whether native or targeted, is capable of transfecting or transducing.

- 15 "Secondary target site" refers to a second site which a vector, whether native or targeted, is capable of transfecting or transducing.

"Target cell" simply refers to a cell which a vector, whether native or targeted, is capable of transfecting or transducing.

- 20 "Primary target cell" refers to a first cell which a vector, whether native or targeted, is capable of transfecting or transducing.

"Secondary target cell" refers to a second cell which a vector, whether native or targeted, is capable of transfecting or transducing.

25

The preferred, adenoviral primary vector according to the invention is also preferably a targeted vector, in which the tissue tropism of the vector is altered from that of a wild-type adenovirus. Adenoviral vectors can be modified to produce targeted adenoviral vectors for example as described in: Krasnykh *et al* 1996 J. Virol 70: 6839-6846;

- 30 Wickham *et al* 1996 J. Virol 70: 6831-6838; Stevenson *et al* 1997 J. Virol 71: 4782-

4790; Wickham *et al* 1995 Gene Therapy 2: 750-756; Douglas *et al* 1997 Neuromuscul. Disord 7:284-298; Wickham *et al* 1996 Nature Biotechnology 14: 1570-1573.

5 Primary target cells for the vector system according to the invention include haematopoietic cells (including monocytes, macrophages, lymphocytes, granulocytes or progenitor cells of any of these); endothelial cells; tumour cells; stromal cells; astrocytes or glial cells; muscle cells; and epithelial cells.

10 Thus, a primary target cell according to the invention, capable of producing the second viral vector, may be of any of the above cell types.

15 In a preferred embodiment, the primary target cell according to the invention is a monocyte or macrophage transduced by a defective adenoviral vector containing a first transcription unit for a retroviral *gag-pol* and a second transcription unit capable of producing a packageable defective retroviral genome. In this case at least the second transcription unit is preferably under the control of a promoter-enhancer which is preferentially active in a diseased location within the body such as an ischaemic site or the micro-environment of a solid tumour.

20 In a particularly preferred embodiment, the second transcription unit is constructed such that on insertion of the genome into the secondary target cell, an intron is generated which serves to reduce expression of a viral essential element, such as the viral *env* gene, and permit efficient expression of a therapeutic and/or diagnostic NOI or NOIs.

25 The packaging cell may be an *in vivo* packaging cell in the body of an individual to be treated or it may be a cell cultured *in vitro* such as a tissue culture cell line. Suitable cell lines include mammalian cells such as murine fibroblast derived cell lines or human cell lines. Preferably the packaging cell line is a human cell line, such as for example: HEK293, 293-T, TE671, HT1080.

Alternatively, the packaging cell may be a cell derived from the individual to be treated such as a monocyte, macrophage, blood cell or fibroblast. The cell may be isolated from an individual and the packaging and vector components administered *ex vivo* followed by re-administration of the autologous packaging cells. Alternatively the  
5 packaging and vector components may be administered to the packaging cell *in vivo*. Methods for introducing retroviral packaging and vector components into cells of an individual are known in the art. For example, one approach is to introduce the different DNA sequences that are required to produce a retroviral vector particle e.g. the *env* coding sequence, the *gag-pol* coding sequence and the defective retroviral genome into  
10 the cell simultaneously by transient triple transfection (Landau & Littman 1992 J. Virol. 66, 5110; Soneoka *et al* 1995 Nucleic Acids Res 23:628-633).

The secondary viral vectors may also be targeted vectors. For retroviral vectors, this may be achieved by modifying the Env protein. The Env protein of the retroviral  
15 secondary vector needs to be a non-toxic envelope or an envelope which may be produced in non-toxic amounts within the primary target cell, such as for example a MMLV amphotropic envelope or a modified amphotropic envelope. The safety feature in such a case is preferably the deletion of regions or sequence homology between retroviral components.

20

Preferably the envelope is one which allows transduction of human cells. Examples of suitable *env* genes include, but are not limited to, VSV-G, a MLV amphotropic *env* such as the 4070A *env*, the RD114 feline leukaemia virus *env* or haemagglutinin (HA) from an influenza virus. The Env protein may be one which is capable of binding to a  
25 receptor on a limited number of human cell types and may be an engineered envelope containing targeting moieties. The *env* and *gag-pol* coding sequences are transcribed from a promoter and optionally an enhancer active in the chosen packaging cell line and the transcription unit is terminated by a polyadenylation signal. For example, if the packaging cell is a human cell, a suitable promoter-enhancer combination is that from the  
30 human cytomegalovirus major immediate early (hCMV-MIE) gene and a polyadenylation

signal from SV40 virus may be used. Other suitable promoters and polyadenylation signals are known in the art.

5 The secondary target cell population may be the same as the primary target cell population. For example delivery of a primary vector of the invention to tumour cells leads to replication and generation of further vector particles which can transduce further tumour cells.

10 Alternatively, the secondary target cell population may be different from the primary target cell population. In this case the primary target cells serve as an endogenous factory within the body of the treated individual and produce additional vector particles which can transduce the secondary target cell population. For example, the primary target cell population may be haematopoietic cells transduced by the primary vector *in vivo* or *ex vivo*. The primary target cells are then delivered to or migrate to a site within  
15 the body such as a tumour and produce the secondary vector particles, which are capable of transducing for example mitotically active tumour cells within a solid tumour.

The retroviral vector particle according to the invention will also be capable of transducing cells which are slowly-dividing, and which non-lentiviruses such as MLV  
20 would not be able to efficiently transduce. Slowly-dividing cells divide once in about every three to four days including certain tumour cells. Although tumours contain rapidly dividing cells, some tumour cells especially those in the centre of the tumour, divide infrequently. Alternatively the target cell may be a growth-arrested cell capable of undergoing cell division such as a cell in a central portion of a tumour mass or a stem  
25 cell such as a haematopoietic stem cell or a CD34-positive cell. As a further alternative, the target cell may be a precursor of a differentiated cell such as a monocyte precursor, a CD33-positive cell, or a myeloid precursor. As a further alternative, the target cell may be a differentiated cell such as a neuron, astrocyte, glial cell, microglial cell, macrophage, monocyte, epithelial cell, endothelial cell, hepatocyte, spermatocyte,  
30 spermatid or spermatozoa. Target cells may be transduced either *in vitro* after isolation from a human individual or may be transduced directly *in vivo*.

The invention permits the localised production of high titres of defective retroviral vector particles *in vivo* at or near the site at which action of a therapeutic protein or proteins is required with consequent efficient transduction of secondary target cells. This is more  
5 efficient than using either a defective adenoviral vector or a defective retroviral vector alone.

The invention also permits the production of retroviral vectors such as MMLV-based vectors in non-dividing and slowly-dividing cells *in vivo*. It had previously been possible  
10 to produce MMLV-based retroviral vectors only in rapidly dividing cells such as tissue culture-adapted cells proliferating *in vitro* or rapidly dividing tumour cells *in vivo*. Extending the range of cell types capable of producing retroviral vectors is advantageous for delivery of genes to the cells of solid tumours, many of which are dividing slowly, and for the use of non-dividing cells such as endothelial cells and cells of various  
15 haematopoietic lineages as endogenous factories for the production of therapeutic protein products.

The delivery of one or more therapeutic genes by a vector system according to the present invention may be used alone or in combination with other treatments or  
20 components of the treatment.

For example, the retroviral vector of the present invention may be used to deliver one or more NOI(s) useful in the treatment of the disorders listed in WO-A-98/05635. For ease of reference, part of that list is now provided: cancer, inflammation or inflammatory  
25 disease, dermatological disorders, fever, cardiovascular effects, haemorrhage, coagulation and acute phase response, cachexia, anorexia, acute infection, HIV infection, shock states, graft-versus-host reactions, autoimmune disease, reperfusion injury, meningitis, migraine and aspirin-dependent anti-thrombosis; tumour growth, invasion and spread, angiogenesis, metastases, malignant, ascites and malignant pleural effusion;  
30 cerebral ischaemia, ischaemic heart disease, osteoarthritis, rheumatoid arthritis, osteoporosis, asthma, multiple sclerosis, neurodegeneration, Alzheimer's disease,



atherosclerosis, stroke, vasculitis, Crohn's disease and ulcerative colitis; periodontitis, gingivitis; psoriasis, atopic dermatitis, chronic ulcers, epidermolysis bullosa; corneal ulceration, retinopathy and surgical wound healing; rhinitis, allergic conjunctivitis, eczema, anaphylaxis; restenosis, congestive heart failure, endometriosis, atherosclerosis  
5 or endosclerosis.

In addition, or in the alternative, the retroviral vector of the present invention may be used to deliver one or more NOI(s) useful in the treatment of disorders listed in WO-A-98/07859. For ease of reference, part of that list is now provided: cytokine and cell  
10 proliferation/differentiation activity; immunosuppressant or immunostimulant activity (e.g. for treating immune deficiency, including infection with human immune deficiency virus; regulation of lymphocyte growth; treating cancer and many autoimmune diseases, and to prevent transplant rejection or induce tumour immunity); regulation of haematopoiesis, e.g. treatment of myeloid or lymphoid diseases; promoting growth of  
15 bone, cartilage, tendon, ligament and nerve tissue, e.g. for healing wounds, treatment of burns, ulcers and periodontal disease and neurodegeneration; inhibition or activation of follicle-stimulating hormone (modulation of fertility); chemotactic/chemokinetic activity (e.g. for mobilising specific cell types to sites of injury or infection); haemostatic and thrombolytic activity (e.g. for treating haemophilia and stroke); antiinflammatory activity  
20 (for treating e.g. septic shock or Crohn's disease); as antimicrobials; modulators of e.g. metabolism or behaviour; as analgesics; treating specific deficiency disorders; in treatment of e.g. psoriasis, in human or veterinary medicine.

In addition, or in the alternative, the retroviral vector of the present invention may be  
25 used to deliver one or more NOI(s) useful in the treatment of disorders listed in WO-A-98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune response, including a response not associated with inflammation; inhibit the ability of  
30 macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reaction

and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididymo-orchitis, infertility, orchidial trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillain-Barre syndrome, Sydenham chora, myasthenia gravis,

pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplantation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

Further provided according to the invention are methods of controlling production of a therapeutic NOI or NOIs such that the therapeutic NOI or NOIs is/are preferentially expressed in a secondary target cell population and is/are poorly expressed or not expressed at a biologically significant level in a primary target cell.

The present invention also provides a pharmaceutical composition for treating an individual by gene therapy, wherein the composition comprises a therapeutically effective amount of the retroviral vector of the present invention comprising one or more deliverable therapeutic and/or diagnostic NOI(s) or a viral particle produced by or obtained from same. The pharmaceutical composition may be for human or animal usage. Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular individual.

The composition may optionally comprise a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical

practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

5

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules  
10 either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example  
15 enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

In a further aspect of the present invention, there is provided a hybrid viral vector system  
20 in the general sense (i.e. not necessarily limited to the aforementioned first aspect of the present invention as defined above) for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell.

25

With this particular embodiment, the genetic vector of the invention is thus a hybrid viral vector system for gene delivery which is capable of generation of defective infectious particles from within a target cell. Thus a genetic vector of the invention consists of a primary vector manufactured *in vitro* which encodes the genes necessary to produce a  
30 secondary vector *in vivo*. In use, the secondary vector carries one or more selected genes

for insertion into the secondary target cell. The selected genes may be one or more marker genes and/or therapeutic genes. Marker genes encode selectable and/or detectable proteins.

5 More aspects concerning this particular aspect of the present invention now follow - which teachings are also applicable to the aforementioned aspects of the present invention.

In another aspect the invention provides target cells infected by the primary viral vector or vectors and capable of producing infectious secondary viral vector particles.

10 In a further aspect the invention provides a method of treatment of a human or non-human mammal, which method comprises administering a hybrid viral vector system or target cells infected by the primary viral vector or vectors, as described herein.

15 The primary viral vector or vectors may be a variety of different viral vectors, such as retroviral, adenoviral, herpes virus or pox virus vectors, or in the case of multiple primary viral vectors, they may be a mixture of vectors of different viral origin. In whichever case, the primary viral vectors are preferably defective in that they are incapable of independent replication. Thus, they are capable of entering a target cell and delivering the secondary vector sequences, but not of replicating so as to go on to infect further target cells.

20 In the case where the hybrid viral vector system comprises more than one primary vector to encode the secondary vector, both or all three primary vectors will be used to infect a primary target cell population, usually simultaneously. Preferably, there is a single primary viral vector which encodes all components of the secondary viral vector.

25 The preferred single or multiple primary viral vectors are adenoviral vectors. Adenovirus vectors have significant advantages over other viral vectors in terms of the titres which can be obtained from *in vitro* cultures. The adenoviral particles are also comparatively stable compared with those of enveloped viruses and are therefore more readily purified and  
30 stored. However, current adenoviral vectors suffer from major limitations for *in vivo* therapeutic use since gene expression from defective adenoviral vectors is only transient.

Because the vector genome does not replicate, target cell proliferation leads to dilution of the vector. Also cells expressing adenoviral proteins, even at a low level, are destroyed by an immunological response raised against the adenoviral proteins.

- 5 The secondary viral vector is preferably a retroviral vector. The secondary vector is produced by expression of essential genes for assembly and packaging of a defective viral vector particle, within the primary target cells. It is defective in that it is incapable of independent replication. Thus, once the secondary retroviral vector has transduced a secondary target cell, it is incapable of spreading by replication to any further target cells.

- 10 The secondary vector may be produced from expression of essential genes for retroviral vector production encoded in the DNA of the primary vector. Such genes may include a gag-pol gene from a retrovirus, an envelope gene from an enveloped virus and a defective retroviral genome containing one or more therapeutic genes. The defective retroviral  
15 genome contains in general terms sequences to enable reverse transcription, at least part of a 5' long terminal repeat (LTR), at least part of a 3'LTR and a packaging signal.

- Importantly, the secondary vector is also safe for *in vivo* use in that incorporated into it are one or more safety features which eliminate the possibility of recombination to produce an  
20 infectious virus capable of independent replication.

- To ensure that it is replication defective the secondary vector may be encoded by a plurality of transcription units, which may be located in a single or in two or more adenoviral or other primary vectors. Thus, there may be a transcription unit encoding the  
25 secondary vector genome, a transcription unit encoding *gag-pol* and a transcription unit encoding *env*. Alternatively, two or more of these may be combined. For example, nucleic acid sequences encoding *gag-pol* and *env*, or *env* and the genome, may be combined in a single transcription unit. Ways of achieving this are known in the art.

- 30 Transcription units as described herein are regions of nucleic acid containing coding sequences and the signals for achieving expression of those coding sequences

independently of any other coding sequences. Thus, each transcription unit generally comprises at least a promoter, an enhancer and a polyadenylation signal. The promoter and enhancer of the transcription units encoding the secondary vector are preferably strongly active, or capable of being strongly induced, in the primary target cells under conditions for production of the secondary viral vector. The promoter and/or enhancer may be constitutively efficient, or may be tissue or temporally restricted in their activity. Examples of suitable tissue restricted promoters/enhancers are those which are highly active in tumour cells such as a promoter/enhancer from a MUC1 gene, a CEA gene or a 5T4 antigen gene. Examples of temporally restricted promoters/enhancers are those which are responsive to ischaemia and/or hypoxia, such as hypoxia response elements or the promoter/enhancer of a grp78 or a grp94 gene. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

Hypoxia or ischaemia regulatable expression of secondary vector components may be particularly useful under certain circumstances. Hypoxia is a powerful regulator of gene expression in a wide range of different cell types and acts by the induction of the activity of hypoxia-inducible transcription factors such as hypoxia inducible factor-1 (HIF-1; Wang & Semenza (1993). *Proc. Natl. Acad. Sci USA* 90:430), which bind to cognate DNA recognition sites, the hypoxia-responsive elements (HREs) on various gene promoters. Dachs *et al* (1997). *Nature Med.* 5: 515.) have used a multimeric form of the HRE from the mouse phosphoglycerate kinase-1 (PGK-1) gene (Firth *et al.* (1994). *Proc. Natl. Acad. Sci USA* 91:6496-6500) to control expression of both marker and therapeutic genes by human fibrosarcoma cells in response to hypoxia *in vitro* and within solid tumours *in vivo* (Dachs *et al* *ibid*). Alternatively, the fact that marked glucose deprivation is also present in ischaemic areas of tumours can be used to activate heterologous gene expression specifically in tumours. A truncated 632 base pair sequence of the grp 78 gene promoter, known to be activated specifically by glucose deprivation, has also been shown to be capable of driving high level expression of a reporter gene in murine tumours *in vivo* (Gazit G, *et al* (1995). *Cancer Res.* 55:1660).

Safety features which may be incorporated into the hybrid viral vector system are described below. One or more such features may be present.

5 Firstly, sequence homology between the sequences encoding the components of the secondary vector may be avoided by deletion of regions of homology. Regions of homology allow genetic recombination to occur. In a particular embodiment, three transcription units are used to construct a secondary retroviral vector. A first transcription unit contains a retroviral *gag-pol* gene under the control of a non-retroviral promoter and enhancer. A second transcription unit contains a retroviral *env* gene under the control of a non-retroviral promoter and enhancer. A third transcription unit comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. In the native retroviral genome, the packaging signal is located such that part of the *gag* sequence is required for proper functioning. Normally when retroviral vector systems are constructed therefore, the packaging signal, including part of the *gag* gene, remains in the vector genome. In the present case however, the defective retroviral genome contains a minimal packaging signal which does not contain sequences homologous to *gag* sequences in the first transcription unit. Also, in retroviruses, for example Moloney Murine Leukaemia virus (MMLV), there is a small region of overlap between the 3' end of the *pol* coding sequence and the 5' end of *env*. The corresponding region of homology between the first and second transcription units may be removed by altering the sequence of either the 3' end of the *pol* coding sequence or the 5' end of *env* so as to change the codon usage but not the amino acid sequence of the encoded proteins.

25 Secondly, the possibility of replication competent secondary viral vectors may be avoided by pseudotyping the genome of one retrovirus with the envelope protein of another retrovirus or another enveloped virus so that regions of homology between the *env* and *gag-pol* components are avoided. In a particular embodiment the retroviral vector is constructed from the following three components. The first transcription unit contains a retroviral *gag-pol* gene under the control of a non-retroviral promoter and enhancer. The second transcription unit contains the *env* gene from the alternative enveloped virus, under the control of a non-retroviral promoter and enhancer. The third transcription unit



comprises a defective retroviral genome under the control of a non-retroviral promoter and enhancer. The defective retroviral genome contains a minimal packaging signal which does not contain sequences homologous to *gag* sequences in the first transcription unit.

- 5 Pseudotyping may involve for example a retroviral genome based on a lentivirus such as an HIV or equine infectious anaemia virus (EIAV) and the envelope protein may for example be the amphotropic envelope protein designated 4070A. Alternatively, the retroviral genome may be based on MMLV and the envelope protein may be a protein from another virus which can be produced in non-toxic amounts within the primary target cell such as an
- 10 Influenza haemagglutinin or vesicular stomatitis virus G protein. In another alternative, the envelope protein may be a modified envelope protein such as a mutant or engineered envelope protein. Modifications may be made or selected to introduce targeting ability or to reduce toxicity or for another purpose.
- 15 Thirdly, the possibility of replication competent retroviruses can be eliminated by using two transcription units constructed in a particular way. The first transcription unit contains a *gag-pol* coding region under the control of a promoter-enhancer active in the primary target cell such as a hCMV promoter-enhancer or a tissue restricted promoter-enhancer. The second transcription unit encodes a retroviral genome RNA capable of being packaged
- 20 into a retroviral particle. The second transcription unit contains retroviral sequences necessary for packaging, integration and reverse transcription and also contains sequences coding for an *env* protein of an enveloped virus and the coding sequence of one or more therapeutic genes.
- 25 In a preferred embodiment the hybrid viral vector system according to the invention comprises single or multiple adenoviral primary vectors which encodes or encode a retroviral secondary vector. Adenoviral vectors for use in the invention may be derived from a human adenovirus or an adenovirus which does not normally infect humans. Preferably the vectors are derived from Adenovirus Type 2 or adenovirus Type 5 (Ad2 or
- 30 Ad5) or a mouse adenovirus or an avian adenovirus such as CELO virus (Cotton et al 1993 J. Virol. 67:3777-3785). The vectors may be replication competent adenoviral vectors but

are more preferably defective adenoviral vectors. Adenoviral vectors may be rendered defective by deletion of one or more components necessary for replication of the virus. Typically, each adenoviral vector contains at least a deletion in the E1 region. For production of infectious adenoviral vector particles, this deletion may be complemented by passage of the virus in a human embryo fibroblast cell line such as human 293 cell line, containing an integrated copy of the left portion of Ad5, including the E1 gene. The capacity for insertion of heterologous DNA into such vectors can be up to approximately 7kb. Thus such vectors are useful for construction of a system according to the invention comprising three separate recombinant vectors each containing one of the essential transcription units for construction of the retroviral secondary vector.

Alternative adenoviral vectors are known in the art which contain further deletions in other adenoviral genes and these vectors are also suitable for use in the invention. Several of these second generation adenoviral vectors show reduced immunogenicity (eg E1 + E2 deletions Gorziglia et al 1996 J. Virol. 70: 4173-4178; E1 + E4 deletions Yeh et al 1996 J. Virol. 70: 559-565). Extended deletions serve to provide additional cloning capacity for the introduction of multiple genes in the vector. For example a 25 kb deletion has been described (Lieber et al. 1996 J. Virol. 70: 8944-8960) and a cloning vector deleted of all viral genes has been reported (Fisher et al 1996 Virology 217: 11-22.) which will permit the introduction of more than 35kb of heterologous DNA. Such vectors may be used to generate an adenoviral primary vector according to the invention encoding two or three transcription units for construction of the retroviral secondary vector.

Embodiments of the invention described solve one of the major problems associated with adenoviral and other viral vectors, namely that gene expression from such vectors is transient. The retroviral particles generated from the primary target cells can infect secondary target cells and gene expression in the secondary target cells is stably maintained because of the integration of the retroviral vector genome into the host cell genome. The secondary target cells do not express significant amounts of viral protein antigens and so are less immunogenic than the cells transduced with adenoviral vector.

The use of a retroviral vector as the secondary vector is also advantageous because it allows a degree of cellular discrimination, for instance by permitting the targeting of rapidly dividing cells. Furthermore, retroviral integration permits the stable expression of therapeutic genes in the target tissue, including stable expression in proliferating target  
5 cells.

Preferably, the primary viral vector preferentially infects a certain cell type or cell types. More preferably, the primary vector is a targeted vector, that is it has a tissue tropism which is altered compared to the native virus, so that the vector is targeted to particular  
10 cells. The term "targeted vector" is not necessarily linked to the term "target cell". "Target cell" simply refers to a cell which a vector, whether native or targeted, is capable of infecting or transducing.

The preferred, adenoviral primary vector according to the invention is also preferably a  
15 targeted vector, in which the tissue tropism of the vector is altered from that of a wild-type adenovirus. Adenoviral vectors can be modified to produce targeted adenoviral vectors for example as described in Krasnykh et al. 1996 J. Virol 70: 6839-6846; Wickham et al 1996 J. Virol 70: 6831-6838; Stevenson et al. 1997 J. Virol. 71: 4782-4790; Wickham et al. 1995 Gene Therapy 2: 750-756; Douglas et al. 1997 Neuromuscul. Disord. 7:284-298;  
20 Wickham et al. 1996 Nature Biotechnology 14: 1570-1573.

Primary target cells for the vector system according to the invention include but are not limited to haematopoietic cells (including monocytes, macrophages, lymphocytes, granulocytes or progenitor cells of any of these); endothelial cells; tumour cells; stromal  
25 cells; astrocytes or glial cells; muscle cells; and epithelial cells.

Thus, a primary target cell according to the invention, capable of producing the second viral vector, may be of any of the above cell types. In a preferred embodiment, the primary target cell according to the invention is a monocyte or macrophage infected by a defective  
30 adenoviral vector containing a first transcription unit for a retroviral gag-pol and a second transcription unit capable of producing a packageable defective retroviral genome. In this

case at least the second transcription unit is preferably under the control of a promoter-enhancer which is preferentially active in a diseased location within the body such as an ischaemic site or the micro-environment of a solid tumour. In a particularly preferred embodiment of this aspect of the invention, the second transcription unit is constructed such that on insertion of the genome into the secondary target cell, an intron is generated which serves to reduce expression of the viral *env* gene and permit efficient expression of a therapeutic gene.

The secondary viral vectors may also be targeted vectors. For retroviral vectors, this may be achieved by modifying the envelope protein. The envelope protein of the retroviral secondary vector needs to be a non-toxic envelope or an envelope which may be produced in non-toxic amounts within the primary target cell, such as for example a MMLV amphotropic envelope or a modified amphotropic envelope. The safety feature in such a case is preferably the deletion of regions or sequence homology between retroviral components.

The secondary target cell population may be the same as the primary target cell population. For example delivery of a primary vector of the invention to tumour cells leads to replication and generation of further vector particles which can transduce further tumour cells. Alternatively, the secondary target cell population may be different from the primary target cell population. In this case the primary target cells serve as an endogenous factory within the body of the treated individual and produce additional vector particles which can infect the secondary target cell population. For example, the primary target cell population may be haematopoietic cells transduced by the primary vector *in vivo* or *ex vivo*. The primary target cells are then delivered to or migrate to a site within the body such as a tumour and produce the secondary vector particles, which are capable of transducing for example tumour cells within a solid tumour.

The invention permits the localised production of high titres of defective retroviral vector particles *in vivo* at or near the site at which action of a therapeutic protein or proteins is required with consequent efficient transduction of secondary target cells. This is more

efficient than using either a defective adenoviral vector or a defective retroviral vector alone.

5 The invention also permits the production of retroviral vectors such as MMLV-based vectors in non-dividing and slowly-dividing cells *in vivo*. It had previously been possible to produce MMLV-based retroviral vectors only in rapidly dividing cells such as tissue culture-adapted cells proliferating *in vitro* or rapidly dividing tumour cells *in vivo*. Extending the range of cell types capable of producing retroviral vectors is advantageous for delivery of genes to the cells of solid tumours, many of which are dividing slowly, and  
10 for the use of non-dividing cells such as endothelial cells and cells of various haematopoietic lineages as endogenous factories for the production of therapeutic protein products.

The delivery of one or more therapeutic genes by a vector system according to the  
15 invention may be used alone or in combination with other treatments or components of the treatment. Diseases which may be treated include, but are not limited to: cancer, neurological diseases, inherited diseases, heart disease, stroke, arthritis, viral infections and diseases of the immune system. Suitable therapeutic genes include those coding for  
20 tumour suppressor proteins, enzymes, pro-drug activating enzymes, immunomodulatory molecules, antibodies, engineered immunoglobulin-like molecules, fusion proteins, hormones, membrane proteins, vasoactive proteins or peptides, cytokines, chemokines, anti-viral proteins, antisense RNA and ribozymes.

In a preferred embodiment of a method of treatment according to the invention, a gene  
25 encoding a pro-drug activating enzyme is delivered to a tumour using the vector system of the invention and the individual is subsequently treated with an appropriate pro-drug. Examples of pro-drugs include etoposide phosphate (used with alkaline phosphatase Senter et al., 1988 Proc. Natl. Acad. Sci. 85: 4842-4846); 5-fluorocytosine (with Cytosine deaminase Mullen et al. 1994 Cancer Res. 54: 1503-1506); Doxorubicin-N-p-  
30 hydroxyphenoxyacetamide (with Penicillin-V-Amidase (Kerr et al. 1990 Cancer Immunol. Immunother. 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with

Carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with b-lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli et al. 1988 Proc . Natl. Acad. Sci. 85: 7572-7576) mustard pro-drugs with nitroreductase (Friedlos et al. 1997J Med Chem 40: 1270-1275) and Cyclophosphamide or Ifosfamide  
5 (with a cytochrome P450 Chen et al. 1996 Cancer Res 56: 1331-1340).

Further provided according to the invention are methods of controlling production of a therapeutic gene such that the therapeutic gene is preferentially expressed in the secondary target cell population and is poorly expressed or not expressed at a biologically significant  
10 level in the primary target cell.

In accordance with the invention, standard molecular biology techniques may be used which are within the level of skill in the art. Such techniques are fully described in the literature. See for example; Sambrook *et al* (1989) Molecular Cloning; a laboratory  
15 manual; Hames and Glover (1985 - 1997) DNA Cloning: a practical approach, Volumes I- IV (second edition); Methods for the engineering of immunoglobulin genes are given in McCafferty *et al* (1996) "Antibody Engineering: A Practical Approach".

In summation, the present invention relates to a novel delivery system suitable for  
20 introducing one or more NOIs into a target cell.

In one broad aspect the present invention relates to a retroviral vector comprising a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence  
25 of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector; wherein the retroviral pro-vector comprises a first nucleotide sequence ("NS") capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS;  
30 such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.

In a further broad aspect, the present invention provides a hybrid viral vector system for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell.

Preferably the primary vector is obtainable from or is based on a adenoviral vector and/or the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector.

The invention will now be further described by way of example in which reference is made to the following Figures:

Figure 1 which shows the structure of a retroviral proviral genome;

Figure 2 which shows the addition of a small T splice donor pLTR;

Figure 3 which shows a diagrammatic representation of pL-SA-N;

Figure 4 which shows a diagrammatic representation of pL-SA-N with a splice donor deletion;

Figure 5 which shows the sequence of MLV pICUT;

Figure 6 which shows the insertion of a splice donor at CMV/R junction of EIAV LTR plasmid;

Figure 7 which shows the insertion of a splice acceptor into pEGASUS-1;

Figure 8 which shows the removal of a wild-type splice donor from EIAV vector;

Figure 9 which shows the combination of pCMVLTR+SD with pEGASUS +SA (noSD) to create pEICUT-1;

5 Figure 10 which shows the construction of pEICUT-*LacZ*;

Figure 11 which shows the pEICUT-*LacZ* sequence;

Figure 12 which shows the vector configuration in both transfected and transduced cells;

10

Figure 13 which shows the restriction of gene expression to either packaging or transduced cells;

Figure 14 which shows the construction of a MLV pICUT *Neo*-p450 vector that restricts  
15 hygromycin expression to producer cells and 2B6 (a p450 isoform) expression to transduced cells;

Figure 15 which shows a sequence comparison of mutant *env* (m4070A) with wild type MMLV sequence from the 3' end of the *pol* gene;

20

Figure 16 which shows the complete sequence of the modified *env* gene m4070A;

Figure 17 which shows a restricted gene expression construct; 4070A Envelope to a first cell; p450 to a second cell;

25

Figure 18 which shows the use of an intron to restrict NOI (in this example p450) expression to a transduced cell;

Figure 19 which shows a pictorial representation of the Transfer vector-pElsp1A;

30

Figure 20 which shows a pictorial representation of pElsp1A construct;



Figure 21 which shows a pictorial representation of pE1RevE construct;

Figure 22 which shows a pictorial representation of pE1HORSE3.1- *gagpol* construct;

5

Figure 23 which shows a pictorial representation of pE1PEGASUS4-Genome construct;

Figure 24 which shows a pictorial representation of pCI-*Neo* construct;

10 Figure 25 which shows a pictorial representation of pCI-Rab construct;

Figure 26 which shows a pictorial representation of pE1Rab construct;

15 Figure 27a is a schematic representation of the natural splicing configuration in a retroviral vector;

Figure 27b is a schematic representation of the splicing configuration in known retroviral vectors;

20 Figure 27c is a schematic representation of the splicing configuration according to the present invention; and

Figure 28 is a schematic representation of the dual hybrid viral vector system according to the present invention.

25

In slightly more detail:

Figure 1 shows the structure of a retroviral proviral genome. In this regard, the simplest retroviruses such as the murine oncoretroviruses have three open reading frames; *gag*,  
30 *pol* and *env*. Frameshift during *gag* translation leads to *pol* translation. Env expression and translation is achieved by splicing between the splice donor (SD) and splice acceptor

(SA) shown. The packaging signal is indicated as *Psi* and is only contained in the full length transcripts - not the *env* expressing sub-genomic transcripts where this signal is removed during the splicing event.

5 Figure 2 schematically shows the addition of small T splice donor to pLTR. Here, the small-t splice donor sequence is inserted into an LTR vector downstream of the start of transcription but upstream of *R* sequence such that upon reverse transcription (in the final construct) the U3-splice donor-R cassette is 'inherited' to 5' end of the proviral vector and RNA transcripts expressed contain a splice donor sequence near their 5'  
10 terminus.

Figure 3 shows a schematic diagram of pL-SA-N. Both the consensus splice acceptor (T/C)nNC/TAG-G (Mount 1982 Nucleic Acids Res 10: 459-472) and branch point are shown in underline and bold.. The arrow indicates the intron/exon junction. Here, the  
15 consensus splice acceptor sequence is inserted into the *Stu1/BamH1* sites of pLXSN. By such positioning this acceptor will therefore interact with any upstream splice donor (in the final RNA transcripts).

Figure 4 shows a schematic diagram for the construction of pL-SA-N with a splice donor  
20 deletion. The gT to gC change is made by performing a PCR reaction on the pL-SA-N vector with the two oligonucleotides shown below. The resulting product is then cloned *Spe1-Asc1* into pL-SA-N thus replacing the wild-type splice donor gT with gC. Both *Spe1* and *Asc1* sites are shown in bold and the mutation in the *Spe1* oligonucleotide shown in captial bold.

25

Figure 5 shows the sequence of MLV pICUT.

Figure 6 shows a schematic diagram of the insertion of splice donor at CMV/R junction of ELAV LTR plasmid. PCR is performed with the two oligonulceotides outlined below  
30 and the resulting PCR product cloned *Sac1-BamH1* into CMVLTR with the equivalent piece removed. In the *Sac1* oligonucleotide the arrow indicates the start of transcription,

the new insert is shown in capital with splice donor sequence underlined. The start of R is shown in italics.

Figure 7 shows a schematic diagram of the insertion of splice acceptor into pEGASUS-1.

5 Here, the double stranded oligonucleotide described below is inserted into *Xho*I-*Bpu*1102 digested pEGASUS-1 to generate plasmid pEGASUS+SA. Both consensus splice acceptor (T/C)nNC/TAG-G (Mount 1982 *ibid*) and branch point are shown in underline and bold. The arrow indicates the intron/exon junction.

10 Figure 8 shows a schematic diagram of the removal of wild-type splice donor from EIAV vector. Splice donor sequence removed by overlapping PCR using the oligonucleotides described below and the template pEGASUS+SA. First separate PCR reactions are performed with oligos1+2 and oligos3+4. The resulting amplified products are then eluted and used combined in a third PCR reaction. After 10 cycles of  
15 this third reaction oligo2 and 4 are then added. The resulting product is then cloned *Sac*I-*Sal*I into pEGASUS+SA to create the plasmid pEGASUS+SA(noSD). The position of the splice donor (SD) is indicated. The point mutation changing the wild-type splice donor from GT to GC is shown in bold both in oligo1 and the complementary oligo3.

20

Figure 9 shows a schematic diagram of combining pCMVLTR+SD with pEGASUS+SA(noSD) to create pEICUT-1. Here, one inserts the *Mlu*I fragment of pEGASUS+SA(noSD) into the unique *Mlu*I site of pCMV-LTR.

25 Figure 10 shows a schematic diagram of the construction of pEICUT-LacZ. It is made by the insertion of the *Xho*I-*Bpu*1102 LacZ fragment from pEGASUS-1 and inserting it into the *Xho*I-*Bpu*1102 site of pEICUT-1 as outlined below.

Figure 11 shows the pEICUT-LacZ sequence.

30 Figure 12 shows a schematic diagram of the vector configuration in both transfected and transduced cells. Here, the starting pICUT vector contains no splice donor upstream of

a splice acceptor (in this instance the consensus splice acceptor derived from IgSA) and therefore the resulting RNA transcripts will not be spliced. Thus all transcripts will be full length transcripts containing a packaging signal (A). Upon transduction however the splice donor (in this instance the small-T spliced donor) is 'inherited' to the 5' of the proviral vector such that all RNA transcripts now produced contain splice donor upstream of a splice acceptor i.e. an intron and thus maximal splicing achieved (B).

Figure 13 shows a schematic diagram of the restriction of gene expression to either packaging and transduced cells. Restriction of gene expression in this instance is achieved by placing the hygromycin ORF upstream of the neomycin ORF in MLV pEICUT (a). By this cloning strategy the resulting vector will now express RNA transcripts that express hygromycin only in transfected cells because ribosome 5' cap-dependent translation will read only the upstream ORF efficiently. However upon transduction hygromycin is now contained within a functional intron and is thus deleted from mature transcripts (b) and thus neomycin ORF is now translated in a 5' cap-dependent manner.

Figure 14 shows a schematic diagram of the construction of a MLV pICUT *Neo*-p450 vector that restricts hygromycin expression to producer cells and 2B6 (a p450 isoform) expression to transduced cells. The starting vector for this construction is the pICUT vector of Figure 13 containing both *hygro* and *neo*. The *neo* gene is replaced with the complete p450 2B6 cDNA as follows: The complete 2B6 cDNA is obtained by RT-PCR on human liver RNA (Clontech) using the following primers:

5'ttcgatgatcaccacatggaactcagcgtcctcctcttccttgac3'

5'ttcgagccggctcatcagcggggcaggaagcggatctgggtatgttg3'

This generates the complete 2B6 cDNA with an optimised kosak sequence flanked with unique *Bcl*I and *Ngo*M1 sites. This cDNA is then cloned into the *Bcl*I-*Ngo*M1 site of

pICUT-Hyg-*Neo* thus replacing *Neo* with p450 (see (A) below). Also shown below are the proviral DNA constructs in both transfected (B) and transduced (C) cells.

Figure 15 is a sequence comparison of mutant env (m4070A) with wild type MMLV sequence from the 3' end of the pol gene.

Figure 16 is the complete sequence of altered 4070A.

Figure 17 shows a gene restricted expression retroviral vector whereby the first NOI (the 4070A envelope ORF) is expressed in the initial vector and the second NOI (in this instance p450) is expressed only after vector replication. After replication the 4070A gene is located within a functional intron and thus removed during RNA splicing.

Figure 18 shows a retroviral expression vector whereby the 5' end of the p450 gene (flush to a splice donor) is only found upstream of the 3' end of the p450 gene (flush to SA) after replication and thus only after replication is a functional p450 gene expressed (from spliced mRNA).

## EXAMPLES

### **Example 1 Construction of a split-intron MLV vector .**

(i) Addition of small-T splice donor:

The starting plasmid for this construct is pLXSN (Miller *et al* 1989 *ibid*); Firstly this construct is digested with *Nhe*1 and the backbone re-ligated to create an LTR (U3-R-U5) plasmid. Into this plasmid is then inserted an oligonucleotide containing the splice donor sequence between the *Kpn*1-*Bbe*1 sites. Also contained within this oligonucleotide, downstream of the splice donor is the MLV R sequence up to the *Kpn*1. The resulting plasmid is named 3'LTR-SD (see Figure 2 ).

(ii) Addition of splice acceptor:

The splice acceptor sequence used in this construct (including the branch point- an A residue between 20 and 40 bases upstream of the splice acceptor involved in intron lariat formation (Aebi *et al* 1987 Trends in Genetics 3: 102-107) is derived from an immunoglobulin heavy chain variable region mRNA (Bothwell *et al* 1981 Cell 24: 625-637) but with a consensus/optimised acceptor site. Such a sequence signal is also present in pCI (Promega). This acceptor sequence is firstly inserted into the *Bam*H1-*Stu*I sites of pLXSN as double stranded oligonucleotide to create the vector pL-SA-N (note: SV40 promoter is lost from pLNSX during cloning). See Figure 3 for an outline of the cloning strategy.

(iii) Removal of original splice donor from pL-SA-N.

The removal of the splice donor contained within the *gag* sequence of pL-SA-N is achieved by PCR based site directed mutagenesis. Two oligonucleotides are used to PCR amplify the region spanning the *Asc*I and *Spe*I unique sites of pL-SA-N. Also incorporated in the *Spe*I-spanning oligonucleotide is the agGTaag to agGCaag change also found in the splicing negative pBABE vectors (Morgenstern *et al* 1990 *ibid*). See Figure 4 for cloning strategy outline.

(iv) Combining pL(noSD)-SA-N with 3'LTR-SD.

The pL(noSD)SA-N plasmid contains a normal MLV derived 3'LTR. This is replaced with the 3'LTR-SD sequence by taking the *Nhe*I insert from pL(noSD)SA-N and dropping it into the *Nhe*I digested 3'LTR-SD vector. The resulting plasmid, named pICUT (Intron Created Upon Transduction) contains all the features of this new generation of retroviral vector (see Figure 5 for sequence data)

**Example 2 Construction of a split-intron Lentivector.**

Construction of initial EIAV lentiviral expression vector (also see patent application GB 9727135.7)

5

For the construction of a split-function lentiviral vector the starting point is the vector named pEGASUS-1 (see patent application GB 9727135.7). This vector is derived from infectious proviral EIAV clone pSEIAV19 (accession number: U01866; Payne *et al* 1994). Its construction is outlined as follows: First; the EIAV LTR, amplified by PCR,  
10 is cloned into pBluescript II KS+ (Stratagene). The *MluI/MluI* (216/8124) fragment of pSEIAV19 is then inserted to generate a wild-type proviral clone (pONY2) in pBluescript II KS+ (Figure 1). The *env* region is then deleted by removal of the *Hind* III/*Hind* III fragment to generate pONY2-H. In addition, a *BglII/NcoI* fragment within *pol* (1901/4949) is deleted and a  $\beta$ -galactosidase gene driven by the HCMV IE  
15 enhancer/promoter inserted in its place. This is designated pONY2.10nlsLacZ. To reduce EIAV sequence to 759 base pairs and to drive primary transcript off a CMV promoter: First; sequence encompassing the EIAV polypurine tract (PPT) and the 3'LTR are PCR amplified from pONY2.10LacZ using primers:

20 PPTEIAV + (Y8198): GACTACGACTAGTGTATGTTTAGAAAAACAAGG,  
and

3'NEGSpeI(Y8199): CTAGGCTACTAGTACTGTAGGATCTCGAACAG.

25 The PCR product is then cloned into the *SpeI* site of pBS II KS<sup>+</sup>; orientated such that U5 is proximal to *NorI* in the pBlueScript II KS<sup>+</sup>

Next, for the reporter gene cassette, a CMV promoter/*LacZ* from pONY 2.10nlsLacZ is removed by *PstI* digest and cloned into the *PstI* site of pBS.3'LTR orientated such that  
30 *LacZ* gene is proximal to the 3'LTR, this vector is named pBS CMVLacZ.3'LTR.

The 5' region of the EIAV vector is constructed in the expression vector pCIE<sub>neo</sub> which is derivative of pCIneo (Promega)-modified by the inclusion of approximately 400 base pairs derived from the 5' end of the full CMV promoter as defined previously. This 400 base pair fragment is obtained by PCR amplification using primers:

5

VSAT1: (GGGCTATATGAGATCTTGAATAATAAAATGTGT) and

VSAT2: (TATTAATAACTAGT) and

10 pHIT60 (Soneoka *et al* 1995 Nucleic Acids Res 23: 628-633) as template. The product is digested with *Bgl*III and *Spe*I and cloned into the *Bgl*III/*Spe*I sites of pCIE-Neo.

A fragment of the EIAV genome running from the R region to nt 150 of the *gag* coding region (nt 268 to 675) is amplified from pSEIAV with primers:

15

CMV5'EIAV2:

(Z0591)(GCTACGCAGAGCTCGTTTAGTGAACCGGGCACTCAGATTCTG:

(sequences underlined anneals to the EIAV R region) and

20

3'PSI.NEG (GCTGAGCTCTAGAGTCCTTTTCTTTTACAAAGTTGG).

The resulting PCR product is flanked by *Xba*I and *Sac*I sites. This is then cut and cloned into the pCIE-Neo *Xba*I-*Sac*I sites. The resulting plasmid, termed  
25 pCIE<sub>neo</sub>5'EIAV now contains the start of the EIAV R region at the transcriptional start point of the CMV promoter. The CMV*LacZ*/3LTR cassette is then inserted into the pCIE<sub>neo</sub>5'EIAV plasmid by taking the *Apa*I to *Not*I fragment from pBS.CMV*LacZ*.3LTR and cloning it into the *Sal*I-*Not*I digested pCIE<sub>neo</sub>.5'EIAV (the *Sal*I and *Apa*I sites is T4 "polished" to create blunt ends prior to the vector and  
30 insert respective *Not*I digests). The resulting plasmid is named pEGASUS-1.



For use as a gene delivery vector pEGASUS-1 requires both *gag/pol* and *env* expression provided *in trans* by a packaging cell. For the source of *gag/pol* an EIAV *gagpol* expression plasmid (pONY3) is made by inserting the *Mlu* I/*Mlu* I fragment from pONY2-H into the mammalian expression plasmid pCI-*neo* (Promega) such that the *gag-pol* gene is expressed from the hCMV-MIE promoter-enhancer and contains no LTR sequences. For the source of *env*; the pRV583 VSV-G expression plasmid is routinely used. These three vectors are used in a three plasmid co-transfection as described for MLV-based vectors (Soneoka *et al* 1995 Nucl. Acids Res. 23:628-633) the resulting virus routinely titres at between  $10^4$  and  $10^5$  *lacZ* forming units per ml on D17 fibroblasts.

#### Construction of a EIAV lentiviral version vector of pICUT; named pEICUT

To construct pEICUT firstly pEGASUS-1 the *Xma*I-*SexA*I fragment is removed from pEGASUS-1 and the ends 'blunted' with T4 polymerase and plasmid re-ligated to create a plasmid containing only the CMV-R-U5 part of pEGASUS-1 which retains the SV40-*Neo* cassette in the backbone. This plasmid is named CMVLTR. To insert a splice donor at the CMV-R border PCR is carried out with the two oligonucleotides shown below in Figure 6 and as outlined in the Figure 6 legend. The resulting plasmid is named pCMVLTR+SD. The same immunoglobulin based consensus splice acceptor as for MLV pICUT (see earlier) is used in the EIAV version. This is inserted using oligonucleotides described in Figure 7 into the *Xho*I-*Bpu*1102 site of pEGASUS-1 to create the plasmid pEGASUS+SA. The wild-type splice donor of EIAV is removed by carrying out overlapping PCR with the oligonucleotides and methodology as described in Figure 8, using pEGASUS+SA as a template to generate the plasmid pEGASUS+SA(noSD). To then create pEICUT-1, the *Mlu*I-*Mlu*I fragment from pEGASUS+SA(noSD) is then inserted into the unique *Mlu*I site of pCMVLTR+SD to generate pEICUT-1 (see Figure 9). *LacZ* can be then transferred from pEGASUS-1 into pEICUT-1 by *Xho*I-*Bpu*1102 digest and insertion to create pEICUT-Z (see Figure 10; for sequence data see Figure 11).

Both the MLV and EIAV pICUT vectors contain a strong splice acceptor upstream of the splice donor and therefore no functional intron (introns require splice donors positioned 5' of splice acceptors). For this reason, when the vector is transfected into producer cells the resulting transcripts generated will not be spliced. Thus the packaging signal  
5 will not be lost and as a consequence maximal packaging is achievable (see Figure 12).

However because of the unique way by which retroviruses replicate, upon transduction, transcripts generated from the integrated pICUT vector will differ from those of transfected cells described above. This is because during replication the 3'U3 promoter  
10 (up to the 5' start of R) is copied and used as the 5' promoter in transduced cells. For this reason transcripts generated from integrated pICUT will now contain a strong splice donor 5' of a strong splice acceptor, both of which being located upstream of the *neo* ORF. Such transcripts will therefore contain a functional intron in the 5'UTR (untranslated region) and thus be maximally spliced and translated.

15 Another advantage of such vectors described above is that because the intron is created only upon transduction it is possible to limit gene expression to either packaged or transduced cells. One example of how this is achieved is outlined in Figures 13. The strategy entails the cloning of a second gene (in this example hygromycin) upstream of  
20 the splice acceptor. This is achieved by taking out the hygromycin cDNA on a *SalI* fragment from SelctaVector Hygro (Ingenius; Oxfordshire, UK), and cloning this into a *XhoI* site (located upstream of the splice acceptor) of pICUT. This vector selectively expresses hygromycin in the transfected cells and neomycin in transduced cells. The reason for this is that in any one mRNA transcript only the first gene is translated by the  
25 ribosome without the aid of internal ribosome binding sites (IRESs). In the transfected cell this gene will be hygromycin. However in the transduced cells because the hygromycin open reading frame (ORF) is contained within a functional intron this gene will now be removed from mature mRNA transcripts thus allowing *neo* ORF translation.

30 Vectors with such cell specific gene expression maybe of clinical use for a variety of reasons; By way of example, expression of resistance markers can be restricted to

producer cells- where they are required and not in transduced cells where they may be immunogenic. By way of another example, expression of toxic genes such as ricin and dominant negative signalling proteins could be restricted to transduced cells where they may be required to optionally arrest cell growth or kill cells but not in producer cells-  
5 where such features would prevent high titre virus production. Figure 14 shows a *Neo*-p450 MLV pICUT construct such that only *Neo* is expressed in producer cells and the pro-drug p450 2B6 isoform expressed in transduced cells.

Another benefit of creating an intron upon transduction is that any essential elements  
10 required for vector function can now be placed inside a functional intron, which is created upon transduction, and be removed from transduced cell transcripts. By way of example, with both the MLV and the lentivector pICUT vectors, the viral transcript contained the functional *Psi* packaging signal (see Bender *et al* 1987 for the position of *Psi* in MLV; see patent application GB 9727135.7 for position of *Psi* in EIAV) within an  
15 intron which was created upon transduction and removed from the transduced cell transcripts.

The benefits from such an arrangement include:

20 (i) Enhanced translation from resulting transcripts because ribosomes may "stutter" in the presence of a *Psi* secondary structure- if present (Krall *et al* 1996 *ibid* and reference therein).

(ii) In the absence of the packaging signal, transcript packaging by endogenous  
25 retroviruses is prevented.

(iii) Unwanted premature translation initiation is prevented when viral essential elements such as *gag* (and other potential ATG translation start sites) are removed from the transcripts expressed in transduced cells. This is of particular benefit when packaging  
30 signals extend into *gag* as is the case for both the EIAV and MLV pICUT vectors.

(iv) Promoter, enhancers and suppressors may be placed within an intron created upon transduction thus mimicking other transcript arrangements like those generated from CMV that contain such entities within introns (Chapman *et al* 1991 *ibid*)

5 In summation the novel pICUT vector system described in the present invention facilitates the following arrangements:

- (I) Maximal packaging and reduced translation of transcripts in producer cells.
- 10 (ii) Maximal splicing and therefore intron enhanced translation of transcripts in transduced cells
- (iii) Restriction of gene and/or viral essential element expression to either producer or transduced cells.

15

**Example 3 Construction of an MMLV amphotropic *env* gene with minimal homology to the *pol* gene and a *gag-pol* transcription cassette**

20 In the Moloney murine leukaemia virus (MMLV), the first approximately 60 bps of the *env* coding sequence overlap with sequences at the 3' end of the *pol* gene. The region of homology between these two genes was removed to prevent the possibility of recombination between them in cells expressing both genes.

25 The DNA sequence of the first 60 bps of the coding sequence of *env* was changed while retaining the amino acid sequence of the encoded protein as follows. A synthetic oligonucleotide was constructed to alter the codon usage of the 5'-end of *env* (See Figure 15) and inserted into the remainder of *env* as follows.

30 The starting plasmid for re-construction of the 5' end of the 4070A gene was the pCI plasmid (Promega) into which had previously been cloned the *Xba*1-*Xba*1 fragment containing the 4070A gene from pHIT456 (Soneoka *et al* 1995 *ibid*) to form pCI-4070A.

A PCR reaction was performed with primers A and B (Figure 15) on pCI-4070A to produce a 600 base pair product. This product was then cloned between the *Nhe*I and *Xho*I sites of pCI-4070A. The resulting construct was sequenced across the *Nhe*I/*Xho*I region. Although the amino acid sequence of the resulting gene is the same as the original 4070A, the region of homology with the *pol* gene is removed.

The complete sequence of the modified *env* gene m4070A is given in Figure 16. This sequence is inserted into the expression vector pCI (Promega) by standard techniques. The CMV *gag-pol* transcription unit is obtained from pHIT60 (Soneoka *et al* 1995 *ibid*).

10

#### Example 4 Deletion of *gag* sequences from the retroviral packaging signal.

A DNA fragment containing the LTR and minimal functional packaging signal is obtained from the retroviral vector MFG (Bandara *et al* 1993 Proc Natl Acad Sci 90: 10764-10768) or MMLV proviral DNA by PCR reaction using the following oligonucleotide primers:

*Hind*III: GCATTAAAGCTTTGCTCT

20 L523: GCCTCGAGCAAAAATTCAGACGGA

This PCR fragment contains MMLV nucleotides +1 to +523 and thus does not contain *gag* coding sequences which start at +621 (numbering based on the nucleotide sequence of MMLV Shinnick *et al* 1981 Nature 293: 543-548).

25 The PCR fragment can be used to construct a retroviral genome vector by digestion using *Hind*III and *Xho*I restriction enzymes and sub-cloning using standard techniques. Such vectors contain no homology with *gag* coding sequences.

#### Example 5 Construction of defective retroviral genome

30

The transcription unit capable of producing a defective retroviral genome is shown in Figure 17. It contains the following elements: a hypoxia regulated promoter enhancer comprising 3 copies of the PGK - gene HRE and a SV40 promoter deleted of the 72bp-repeat enhancer from pGL3 (Promega); a MMLV sequence containing R, U5 and the packaging signal; the coding sequence of m4070A (Example 3); a splice acceptor; a cloning site for insertion of a coding sequence for a therapeutic protein; the polypyrimidine tract from MMLV; a second copy of the HRE-containing promoter-enhancer; a splice donor site; and a second copy of R, U5.

On reverse transcription and integration of the vector into the secondary target cell, the splice donor is introduced upstream of the *env* gene causing it to be removed from mRNA by splicing and thereby permitting efficient expression of the therapeutic gene only in the secondary target cell (See Figure 17).

#### Example 6 Construction of a conditional expression vector for Cytochrome P450

Figure 18 shows the structure of retroviral expression vector cDNA coding sequences from the cytochrome P450 gene in two halves such that only upon transduction is the correct splicing achieved to allow P450 expression. This therefore restricts expression to transduced cells.

1) The starting plasmid for the construction of this vector is pLNSX (Miller and Rosman 1989 BioTechniques 7: 980-990). The natural splice donor (...agGTAag...) contained within the packaging signal of pLNSX (position 781/782) is mutated by PCR mutagenesis using the ALTERED SITES II mutagenesis kit (Promega) and a synthetic oligonucleotide of the sequence:

5'-caaccaccgggagGCaagctggccagcaactta-3'

2) A CMV promoter from the pCI expression vector (Promega) is isolated by PCR using the following two oligonucleotides:

Primer 1: 5'-atcggctagcagatcttcaatattggccattagccatat-3'

Primer 2: 5'-atcgagatctgcggccgcttacctgcccagtcctcacgaccaa-3'

5

This produces a fragment containing the CMV promoter with a 5'*Nhe*1 site (Primer 1) and a 3' *Not*1 and *Xba*1 site (Primer 2). It is cut with *Nhe*1 and *Xba*1 and cloned into pLNSX from which an *Nhe*1-*Nhe*1 fragment has been removed.

- 10 3) The 5' end of a cytochrome P450 cDNA coding sequence is isolated by RT-PCR from human liver RNA (Clontech) with the following primers:

Primer 3: 5'-atcggcgccgcccaccatggaactcagcgtcctcctcttccttgaccctagg-3'

15 Primer 4: 5'-atcggcgccgcacttacCtgtgtgccccaggaaagtatttcaagaagccag-3'

- This amplifies the 5' end of the p450 from the ATG to residue 693 (numbering from the translation initiation site Yamano *et al* 1989 Biochem 28:7340-7348). Contained on the 5' end of the fragment (derived from Primer 3) is also a *Not*1 site and an optimised "Kozak" translation initiation signal. Contained on the 3' end of the sequence (derived from primer 4) is another *Not*1 site and a consensus splice donor sequence (also found in pCI and originally derived from the human beta globin gene) with the GT splice donor pair located flush against residue 704 of P450 (the complementary residue is shown in upercase in Primer 4). This fragment is digested with *Not*1 and cloned into the *Not*1 digested plasmid generated in step 2.
- 20
- 25

- 4) The *Nhe*1-*Nhe*1 fragment removed during the cloning of step 2 is then re-introduced into the plasmid of step 3. This creates a retroviral vector as described in Figure 17 but missing the 3' end P450.

30

5) The 3' of the P450 coding sequence is isolated by RT-PCR amplification from human liver RNA (Clontech) using the following primers:

Primer 5: actgtgatcatagggcacctattggttactgacatccactttctccacagGcaagttacaaaacctgc  
aggaaatcaatgcttacatt-3'

Primer 6: actgatcgatttcctcagccccttcagcggggcaggaagc-3'

This generates the PCR amplified 3' end of P450 from residue 705 (in uppercase primer 5) and extends past the translation termination codon. Contained within the 5' end of this product and generated by primer 5 is a *Bcl*I restriction site and a consensus splice acceptor and branch point (also found in pCI and originally from an immunoglobulin gene) upstream of residue 705. Contained at the 3' end of this product downstream of the stop codon and generated by primer 6 is a *Cl*aI site. This PCR product is then digested with *Bcl*I and *Cl*aI and cloned into the vector of step 3 with the *Bcl*I-*Cl*aI fragment removed to generate the retroviral vector as shown in Figure 18.

The following examples describe the construction of an adenolentiviral system that can be used for the transient production of lentivirus *in vitro* or *in vivo*.

#### First Generation Recombinant Adenovirus

The first generation adenovirus vectors consist of a deletion of the E1 and E3 regions of the virus allowing insertion of foreign DNA, usually into the left arm of the virus adjacent to the left Inverted Terminal Repeat (ITR). The viral packaging signal (194-358 nt) overlaps with the E1a enhancer and hence is present in most E1 deleted vectors. This sequence can be translocated to the right end of the viral genome (Hearing & Shenk, 1983 Cell 33: 59-74). Therefore, in an E1 deleted vector 3.2 kb can be deleted (358-3525 nt).

Adenovirus is able to package 105% length of the genome, thus allowing for addition of an extra 2.1 kb. Therefore, in an E1/E3 deleted viral vector the cloning capacity becomes 7-8 kb (2.1 kb + 1.9 kb (removal of E3) + 3.2 kb (removal of E1). Since the recombinant



adenovirus lacks the essential E1 early gene it is unable to replicate in non-E1 complementing cell lines. The 293 cell line was developed by Graham *et al.* (1977 J Gen Virol 36: 59-74) and contains approximately 4 kb from the left end of the Ad5 genome including the ITR, packaging signal, E1a, E1b and pIX. The cells stably express E1a and E1b gene products, but not the late protein IX, even though pIX sequences are within E1b. In non-complementing cells the E1 deleted virus transduces the cell and is transported to the nucleus but there is no expression from the E1 deleted genome.

### First Generation Adenovirus Production System

#### 10 Microbix Biosystems – nbl Gene Sciences

The diagram in Figure 19 shows the general strategy used to create recombinant adenoviruses using the microbix system

15 The general strategy involves cloning the foreign DNA into an E1 shuttle vector, where the E1 region from 402-3328 bp is replaced by the foreign DNA cassette. The recombinant plasmid is then co-transfected into 293 cells with the pJM17 plasmid. pJM17 contains a deletion of the E3 region and an insertion of the prokaryotic pBRX vector (including the ampicillin resistance and bacterial ori sequences) into the E1 region at 3.7 map units. This 40 kb plasmid is therefore too large to be packaged into adeno nucleocapsids but can be propagated in bacteria. Intracellular recombination in 293 cells results in replacement of the *amp<sup>r</sup>* and ori sequences with the insert of foreign DNA.

### Example 7 Construction of Transfer plasmids for the creation of Adenoviruses

#### 25 containing EIAV Components

In order to produce lentiviral vectors four adenovirus need to be made: genome, *gagpol*, envelope (rabies G) and *Rev*. The lentiviral components are expressed from heterologous promoters they contain introns where needed (for high expression of *gagpol*, *Rev* and Rabies envelope) and a polyadenylation signal. When these four viruses are transduced into E1a minus cells the adenoviral components will not be expressed but the heterologous

promoters will allow the expression of the lentiviral components. An example is outlined below (example 1) of the construction of an ELAV adenoviral system (Application number: 9727135.7). The ELAV is based on a minimal system that is one lacking any of the non-essential ELAV encoded proteins (S2, Tat or envelope). The envelope used to pseudotype the ELAV is the rabies envelope (G protein). This has been shown to pseudotype ELAV well (Application number: 9811152.9).

### Transfer Plasmids

Described below is the construction of the transfer plasmids containing the ELAV components. The transfer plasmid is pE1sp1A (Figure 20).

The recombinant transfer plasmids can be used to make recombinant adenoviruses by homologous recombination in 293 cells.

A pictorial representation of the following plasmids is attached.

#### A) pE1RevE – Rev Construct

The plasmid pCI-Rev is cut with *Apa* I and *Cla* I. The 2.3 kb band encoding ELAV Rev is blunt ended with Klenow polymerase and inserted into the *Eco* RV site of pE1sp1A to give plasmid pE1RevE (Figure 21).

#### B) pE1HORSE3.1 – *gagpol* Construct

pHORSE3.1 was cut with *Sna* BI and *Not* I. The 6.1 kb band encoding ELAV *gagpol* was inserted into pE1RevE cut with *Sna* BI and *Not* I (7.5 kb band was purified). This gives plasmid pE1HORSE3.1 (Figure 22).

**C) pE1PEGASUS – Genome Construct**

pEGASUS4 was cut with *Bgl* II and *Not* I. The 6.8 kb band containing the ELAV vector genome was inserted into pE1RevE cut with *Bgl* II and *Not* I (6.7kb band was purified).

5 This gave plasmid pE1PEGASUS (Figure 23).

**D) pCI-Rab – Rabies Construct**

10 In order to make pE1Rab the rabies open reading frame was inserted into pCI-*Neo* (Figure 24) by cutting plasmid pSA91RbG with *Nsi* I and *Ahd* I. The 1.25 kb band was bluntended with T4 DNA polymerase and inserted into pCI-*Neo* cut with *Sma* I. This gave plasmid pCI-Rab (Figure 25).

**F) pE1Rab – Rabies Construct**

15

pCI-Rab was cut with *Sna* BI and *Not* I. The 1.9 b band encoding Rabies envelope was inserted into pE1RevE cut with *Sna* BI and *Not* I (7.5 b band was purified). This gave plasmid pE1Rab (Figure 26).

**20 SUMMARY**

The present invention relates to a novel delivery system suitable for introducing one or more NOIs into a target cell.

25 In one preferred aspect the present invention covers a retroviral vector comprising a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector;

30 wherein the retroviral pro-vector comprises a first nucleotide sequence ("NS") capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS;

such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.

Alternatively expressed, this aspect covers a novel delivery system which comprises one or more NOIs flanked by a functional SD and SA provided that this has been generated from a pro-vector in which the order of the SD and SA is reversed to render the splicing non-functional.

This aspect of the present invention can be called the "split-intron" aspect. A schematic diagram showing this aspect of the present invention is provided in Figure 27c. In contrast, Figures 27a and 27b show splicing configurations in known retroviral vectors.

Another broad aspects of the present invention include a novel delivery system which comprises one or more adenoviral vector components capable of packaging one or more lentiviral vector components, wherein optionally the lentiviral vector comprises a split intron configuration.

This aspect of the present invention in the general sense can be called a hybrid viral vector system. In this particular case, the combination of an adenoviral component and a lentiviral component can be called a dual hybrid viral vector system.

A schematic diagram showing this aspect of the present invention is provided in Figure 28.

These and other broad aspects of the present invention are discussed herein.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as

claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

**ANNEX**  
**CLAIMS**

1. A retroviral vector comprising a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector; wherein the retroviral pro-vector comprises a first nucleotide sequence ("NS") capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS; such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.
2. A retroviral vector according to claim 1 wherein the retroviral pro-vector comprises a third NS that is upstream of the second nucleotide sequence; wherein the third NS is capable of yielding a non-functional splice donor site.
3. A retroviral vector according to claim 1 or claim 2 wherein the retroviral vector further comprises a second NOI; wherein the second NOI is downstream of the functional splice acceptor site.
4. A retroviral vector according to claim 3 wherein the retroviral pro-vector comprises the second NOI; wherein the second NOI is downstream of the second nucleotide sequence.
5. A retroviral vector according to claim 3 or claim 4 wherein the second NOI, or the expression product thereof, is or comprises a therapeutic agent or a diagnostic agent.
6. A retroviral vector according to any one of the preceding claims wherein the first NOI, or the expression product thereof, is or comprises any one or more of an agent conferring selectability (e.g. a marker element), a viral essential element, or a part thereof, or combinations thereof.

7. A retroviral vector according to any one of the preceding claims wherein the first NS is at or near to the 3' end of a retroviral pro-vector; preferably wherein the 3' end comprises a U3 region and an R region; and preferably wherein the first NS is located  
5 between the U3 region and the R region.
8. A retroviral vector according to claim 7 wherein the U3 region and/or the first NS of the retroviral pro-vector comprises an NS that is a third NOI; wherein the NOI is any one or more of a transcriptional control element, a coding sequence or a part  
10 thereof.
9. A retroviral vector according to any one of the preceding claims wherein the first NS is obtainable from a virus.
10. A retroviral vector according to claim 9 wherein the first NS is an intron or a part  
15 thereof.
11. A retroviral vector according to claim 10 wherein the intron is obtainable from the small t-intron of SV40 virus.
12. A retroviral vector according to any one of the preceding claims wherein the retroviral pro-vector comprises a retroviral packaging signal; and wherein the second NS is located downstream of the retroviral packaging signal such that splicing is preventable at a primary target site.  
20
13. A retroviral vector according to any one of the preceding claims wherein the second NS is placed downstream of the first NOI such that the first NOI is capable of being expressed at a primary target site.  
25

14.. A retroviral vector according to any one of the preceding claims wherein the second NS is placed upstream of a multiple cloning site such that one or more additional NOIs may be inserted.

5 15. A retroviral vector according to any one of the preceding claims wherein the second NS is a nucleotide sequence coding for an immunological molecule or a part thereof.

10 16. A retroviral vector according to claim 15 wherein the immunological molecule is an immunoglobulin.

17. A retroviral vector according to claim 16 wherein the second NS is a nucleotide sequence coding for an immunoglobulin heavy chain variable region.

15 18. A retroviral vector according to any one of the preceding claims wherein the vector additionally comprises a functional intron.

19. A retroviral vector according to claim 18 wherein the functional intron is positioned so that it is capable of restricting expression of at least one of the NOIs in a  
20 desired target site.

20. A retroviral vector according to claim 19 wherein the target site is a cell.

21. A retroviral vector according to any one of the preceding claims wherein the  
25 vector or pro-vector is derivable from a murine oncoretrovirus or a lentivirus

22. A retroviral vector according to claim 21 wherein the vector is derivable from MMLV, MSV, MMTV, HIV-1 or EIAV.

30 23. A retroviral vector as defined in any one of the preceding claims wherein the retroviral vector is an integrated provirus.



24. A retroviral particle obtainable from a retroviral vector according to any one of the preceding claims.
- 5 25. A cell transfected or transduced with a retroviral vector according to any one of claims 1-23 or a retroviral particle according to claim 24.
26. A retroviral vector according to any one of claims 1-23 or a viral particle according to claim 24 or a cell according to claim 25 for use in medicine.
- 10 27. Use of a retroviral vector in any one of claims 1 to 23 or a viral particle according to claim 24 or a cell according to claim 25 for the manufacture of a pharmaceutical composition to deliver one or more NOIs to a target site in need of same.
28. A method comprising transfecting or transducing a cell with a retroviral vector  
15 according to any one of claims 1 to 23 or a viral particle according to claim 24 or by use of a cell according to claim 25.
29. A delivery system for a retroviral vector according to any one of claims 1 to 23 or a viral particle according to claim 24 or a cell according to claim 25 wherein the  
20 delivery system comprises one or more non-retroviral expression vector(s), adenoviruse(s), or plasmid(s) or combinations thereof for delivery of an NOI or a plurality of NOIs to a first target cell and a retroviral vector for delivery of an NOI or a plurality of NOIs to a second target cell.
- 25 30. A retroviral pro-vector as defined in any one of the preceding claims.
31. Use of a functional intron to restrict expression of one or more NOIs within a desired target cell.
- 30 32. Use of a reverse transcriptase to deliver a first NS from the 3' end of a retroviral pro-vector to the 5' end of a retroviral vector.

33. A hybrid viral vector system for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell.
34. A hybrid viral vector system according to claim 33 wherein the primary vector is obtainable from or is based on a adenoviral vector and/or the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector.
35. Use of a hybrid viral vector system according to claim 33 and 34 wherein the lentiviral vector has a split-intron configuration.
36. A hybrid viral vector system wherein the lentiviral vector comprises or is capable of delivering a split-intron configuration.
37. A lentiviral vector system wherein the lentiviral vector comprises or is capable of delivering a split-intron configuration.
38. An adenoviral vector system wherein the adenoviral vector comprises or is capable of delivering a split-intron configuration.
39. Vectors or plasmids based on or obtained from any one or more of the entities presented as pE1sp1A, pCI-Neo, pE1RevE, pE1HORSE3.1, pE1PEGASUS4, pCI-Rab, pE1Rab.
40. A hybrid viral vector system for *in vivo* gene delivery, which system comprises a primary viral vector which encodes a secondary viral vector, the primary vector capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell, wherein the primary

vector is obtainable from or is based on a adenoviral vector and the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector.

41. A hybrid viral vector system for *in vivo* gene delivery, which system comprises a primary viral vector which encodes a secondary viral vector, the primary vector capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell, wherein the primary vector is obtainable from or is based on a adenoviral vector and the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector; wherein the viral vector system comprises a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector; wherein the retroviral pro-vector comprises a first nucleotide sequence ("NS") capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS; such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.
42. A retroviral vector capable of differential expression of NOIs in target cells substantially as described herein.

## ANNEX

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**ABSTRACT**  
**VECTOR**

5 A retroviral vector is described. The retroviral vector comprises a functional splice donor site and a functional splice acceptor site; wherein the functional splice donor site and the functional splice acceptor site flank a first nucleotide sequence of interest ("NOI"); wherein the functional splice donor site is upstream of the functional splice acceptor site; wherein the retroviral vector is derived from a retroviral pro-vector;

10 wherein the retroviral pro-vector comprises a first nucleotide sequence ("NS") capable of yielding the functional splice donor site and a second NS capable of yielding the functional splice acceptor site; wherein the first NS is downstream of the second NS; such that the retroviral vector is formed as a result of reverse transcription of the retroviral pro-vector.

15

20

## CLAIMS

1. A modified haematopoietic stem cell (MHSC) comprising at least one expressable nucleotide sequence of interest (NOI) wherein the or each NOI is operably linked to one or more ischaemia like response element (ILRE).  
5
2. A MHSC according to claim 1 in combination with one or more agents that are capable of differentiating the MHSC.
- 10 3. A pharmaceutical composition comprising a MHSC according to claim 1 or claim 2 optionally admixed with a pharmaceutically acceptable diluent, exceipient or carrier..
4. A MHSC according to claim 1 or claim 2 for use in medicine.
- 15 5. A method of expressing one or more NOIs in an ischaemic environment comprising expressing the one or more NOIs of the MHSC according to claim 1 or claim 2 in the ischaemic environment.
6. Use of a MHSC according to claim 1 or claim 2 in the manufacture of a  
20 medicament to treat a condition associated with or caused by ischaemia.
7. A process of treating an individual in need of same comprising administering a MHSC according to claim 1 or claim 2, or a pharmaceutical composition according to claim 3, and allowing expression of one or more of the one or more NOIs.  
25
8. Vectors or plasmids basd on or obtained from any one or more of the entities presented as pE1sp1A, pCI-Neo, pE1RevE, pE1HORSE3.1, pE1PEGASUS4, pCI-Rab, pE1Rab.
- 30 9. A modified HSC (MHSC) which comprises a responsive element that is operable in a macrophage.

10. A modified cell comprising a responsive element that is active in that cell; and an NOI; wherein the modified cell is prepared by transforming a cell by viral transduction with one or more viral vectors wherein at least one of which comprises the NOI and/or the responsive element .
11. A hybrid viral vector system for *in vivo* gene delivery, which system comprises one or more primary viral vectors which encode a secondary viral vector, the primary vector or vectors capable of infecting a first target cell and of expressing therein the secondary viral vector, which secondary vector is capable of transducing a secondary target cell, wherein the primary viral vector and/or the secondary viral vector comprises an ILRE of the present invention.
12. A hybrid viral vector system according to claim 11 wherein the primary vector is obtainable from or is based on a adenoviral vector and/or the secondary viral vector is obtainable from or is based on a retroviral vector preferably a lentiviral vector.
13. Use of an adenoviral vector to deliver an ILRE regulated gene to any cell type for use in any disease.
14. Use of a retroviral vector to deliver an ILRE regulated gene to any cell type for use in any disease.
15. Use of a combination of adenoviral and lentiviral vector to deliver an ILRE regulated gene to any cell type for use in the treatment of a disease.
16. A modified differentiated cell (preferably terminally differentiated cell) comprising at least one expressable nucleotide sequence of interest (NOI) wherein the or each NOI is operably linked to one or more responsive element(s) active in that differentiated cell.

17. One or more adenoviral vector constructs that show low basal activity in normal tissue but are strongly induced under ischaemic conditions.
18. One or more lentiviral vector constructs that show low basal activity in normal  
5 tissue but are strongly induced under ischaemic conditions.
19. One or more of a combination of adenoviral and lentiviral constructs that show low basal activity in normal tissue but are strongly induced under ischaemic conditions.
- 10 20. One or more novel vectors or constructs or promoters or regulatory elements as defined herein.

Figure 1

	<u>HRE's</u>
hEPO	GGGCCCTACGTGCTGTCTCACACAGC
mEPO	GGGCCCTACGTGCTGCCTCGCATGGC
mPGK	CGCGTCGTGCAGGACGTGACAAAT
mLDH	CCAGCGGACGTGCGGGAACCCACGTGTAGG
Glucose trpt	TCCACAGGCGTGCCGTCTGACACGCA
hVEGF	CCACAGTGATACGTGGGCTCCAACAGGTCCTCTT
rVEGF	ACAGTGCATACGTGGGCTTCCACA
hNOS	ACTACGTGCTGCCTAGGG
hAldolase	CCCCTCGGACGTGACTCGGACCACAT
hEnolase	ACGCTGAGTGCGTGCGGGACTCGGAGTACGTGACGGA
mHeme Oxygenase	CGGACGTGCTGGCGTGGCACGTCCTCTC



Figure 2

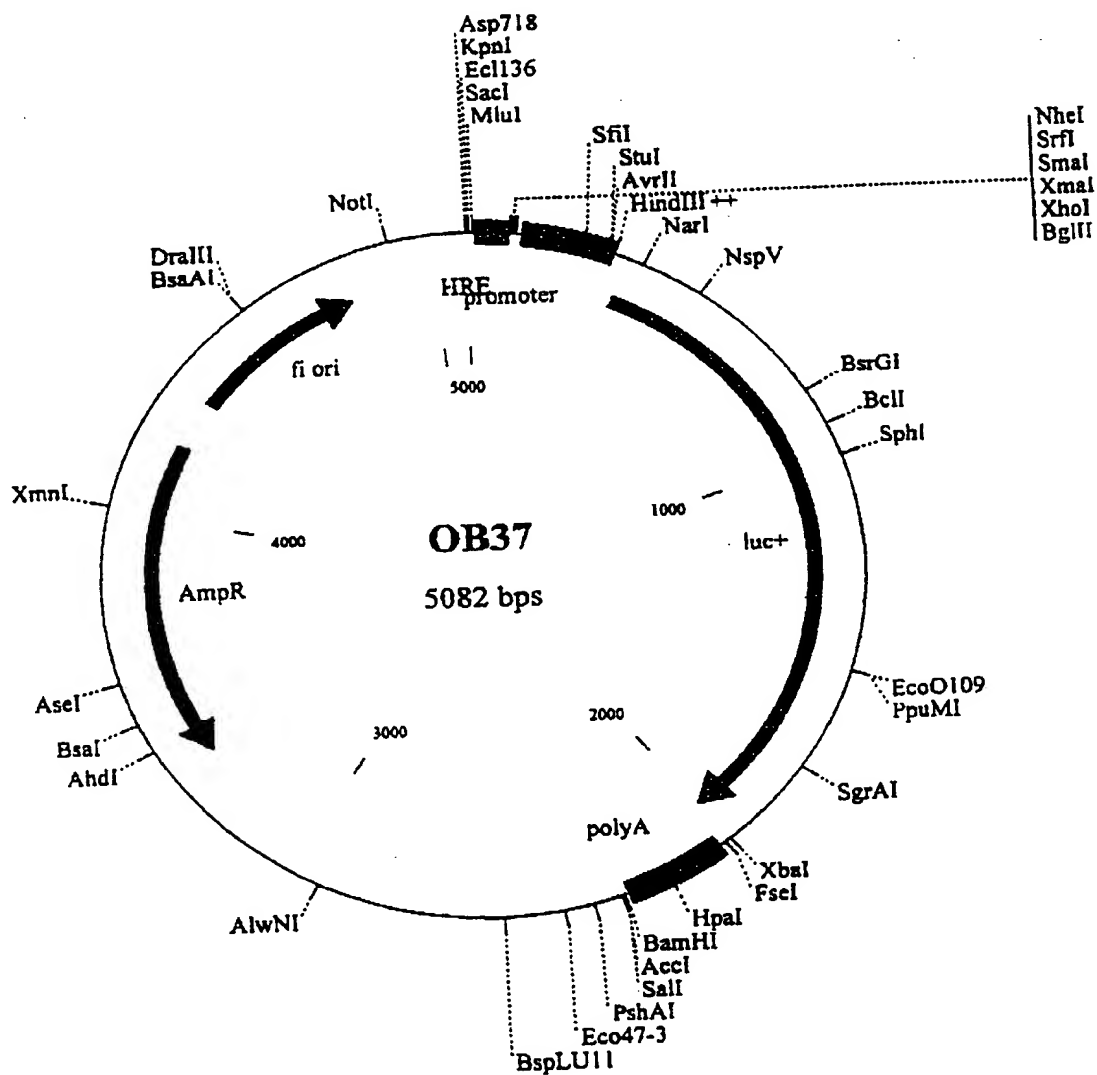


Figure 3.

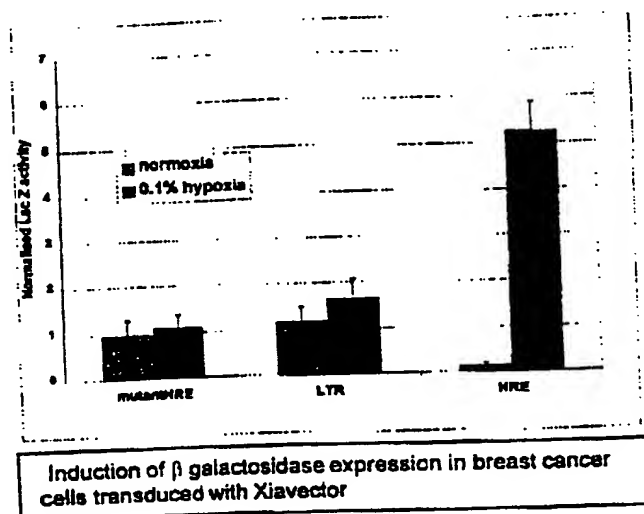


Figure 4A

PGK (PGK x 3 in fig 1a).

As above in the reverse orientation to the natural configuration.

CTAGCTGTCACGTCCCTGCACGACACTAGATGTCACGTCCCTGCACGACA

HRE

HRE

CTAGATGTCACGTCCCTGCACGACTCTAGCCCG

HRE

[REDACTED]

[REDACTED]

[REDACTED]GCAGAGGCCGAGGCCGCCTCGGCCTCTG

Enolase A

Sequence includes three HRE consensus sites spaced as in the native human gene positions -413/-351, first is though to be inactive (Semenza et al JBC 51 32529 1996).

GATCTGAGGGCCGGGACGTGGGGGCCCCAGAGCGACGCTGAGTGCGTGC

(HRE)

HRE

GGGACTCGGAGTTACGTGACGGAGCCCCG

HRE

[REDACTED]

[REDACTED]

[REDACTED]GCAGAGGCCGAGGCCGCCTCGGCCTCTG

**Enolase x 2**

As above but two copies of the synthetic sequence inserted.

GATCTGAGGGCCGGACGTGGGGCCCCAGAGCGACGCTGAGTGCGTGC

(HRE)

HRE

GGGACTCGGAGTACGTGACGGAGCCCCGGATCTGAGGGCCGGACGTG

HRE

(HRE)

GGGCCCCAGAGCGACGCTGAGTGCGTGCGGGACTCGGAGTACGTGAC

HRE

(HRE)

GGAGCCCCG

GCAGAGGCCGAGGCCGC

GCAGAGGCCGAGGCCGC

**CTCGGCCTCTG****Epo x 4**

Tetramer of the human erythropoietin enhancer (positions +3065/3089, (HRE?) marks a site common to LDH that binds a constitutive factor that contributes to hypoxic response, Wang and Semenza PNAS 90 4304 1993)

GATCTGCCCTACGTGCTGTCTCACACAGCCTGGCCCTACGTGCTGTCTC

HRE

(HRE?)

HRE

ACACAGCCTGGATCTGCCCTACGTGCTGTCTCACACAGCCTGGCCCTA

(HRE?)

HRE

(HRE?)

CGTGCTGTCTCACACAGCCTGG

HRE

(HRE?)

GCAGA

GCAGA

**GGCCGAGGCCGCTCGGCCTCTG**

**Note:** In Firth et al the sequence of the complementary strand is described;

**CCAGCGGACGTGCGGGAACCCACGTGTAGG** (-50/-88) written in the conventional 5' to 3' direction with the HRE underlined. An additional regulatory element is highlighted in italics.

GATCTCTACACGTGGGTTCCCGCACGTCCGCTGGGCTCCCACTCTGAC  
(HRE?) HRE CRE

**GTCAGCGG**

**GCAGAGGCCGAGGCCGCC**

TCGGCCTCTG

LDH x 2

As above with 2 copies of the synthetic sequence.

GATCTCTACACGTGGGTTCCTCCGACGTCCGCTGGGCTCCCACTCTGAC  
(HRE?) HRE

GTCAGCGGATCTCTACACGTGGGTTCCCGACAGTCCGCTGGGCTCCCA  
CRE (HRE?) HRE

CTCTGACGTCAGCGG

**CRE**

**GCAGAGGCCGAG**

GGCGCCTCGGCCTCTG

Figure 4B

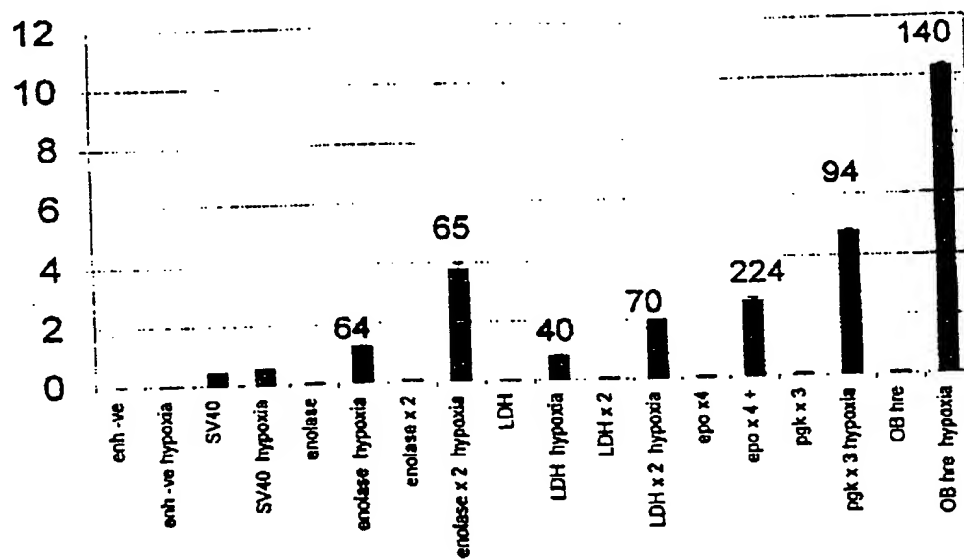


Figure 5

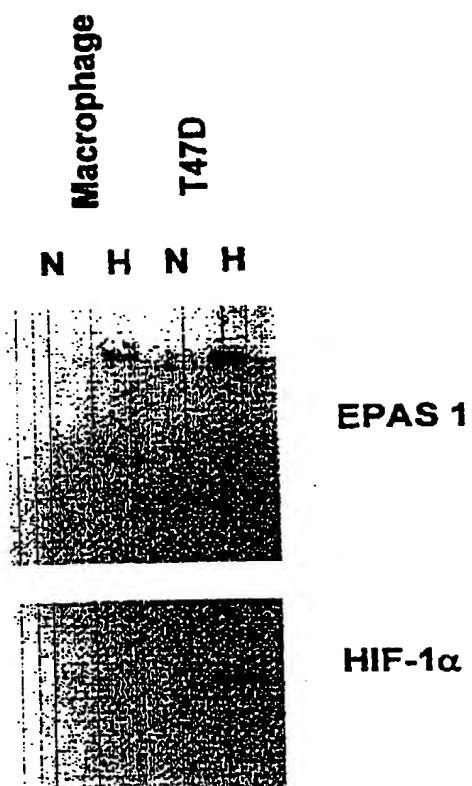


Figure 6

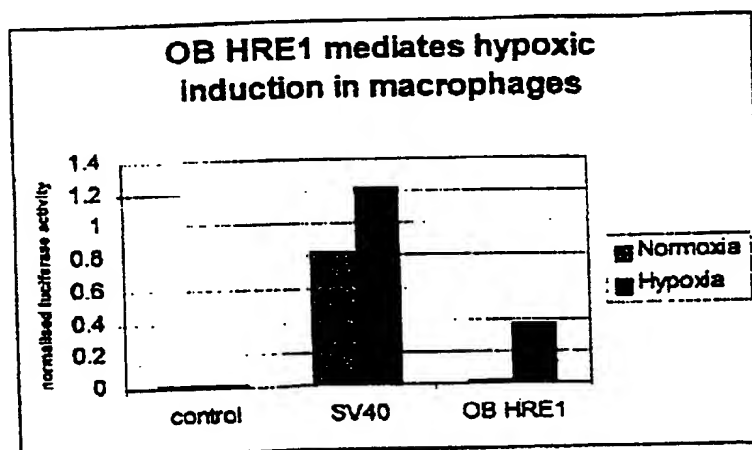




Figure 7

## XiaMac Sequence

GCTAGAGTCGTGCAGGACGTGACATCTAGTGTCGTGCAGGACGTGACA  
HRE HRE  
TCTAGTGTCGTGCAGGACGTGACAGCTAGCATTCCATCACGTGGCCCG  
HRE NF-IL6  
AGAGAAGCATCCGGAGTACTACAAGGACTGCTGACAGCGAGATTCTA  
CAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGTGTGGCCTGGGCG  
NFkB NFkB SP1 SP1  
GGACTGGGGAGTGGCGAGCCCTCAGATGCTGCATATAAGCAGCTGCTT  
SP1  
TTGCCC

Figure 8

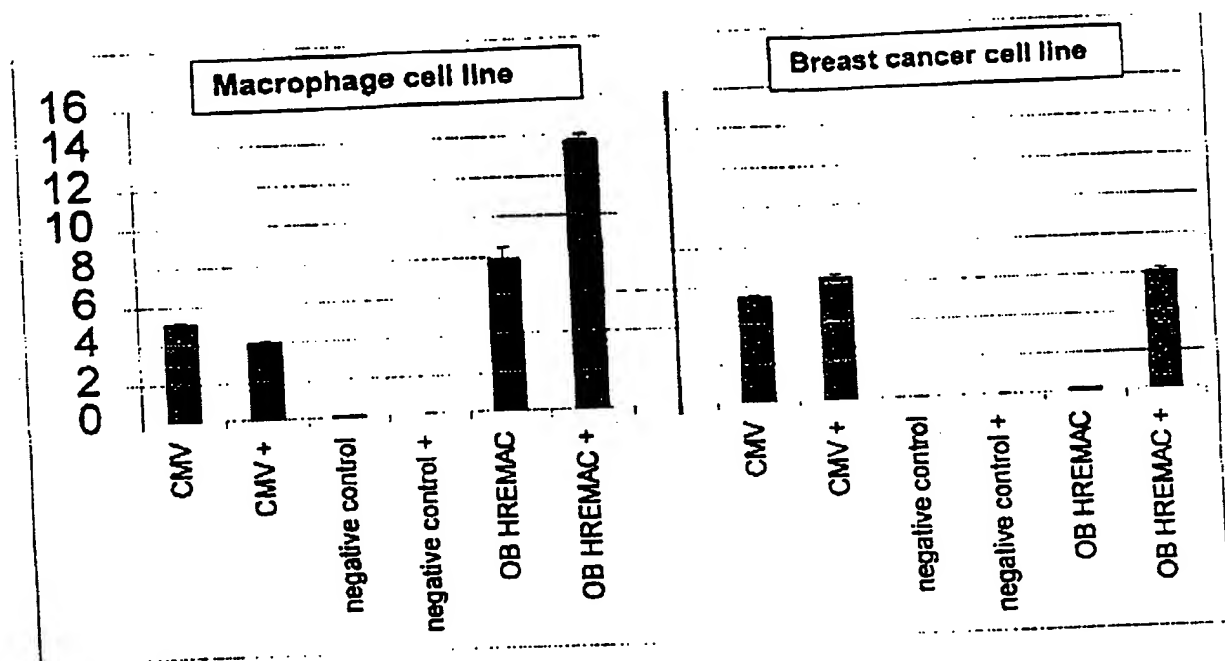


Figure 9

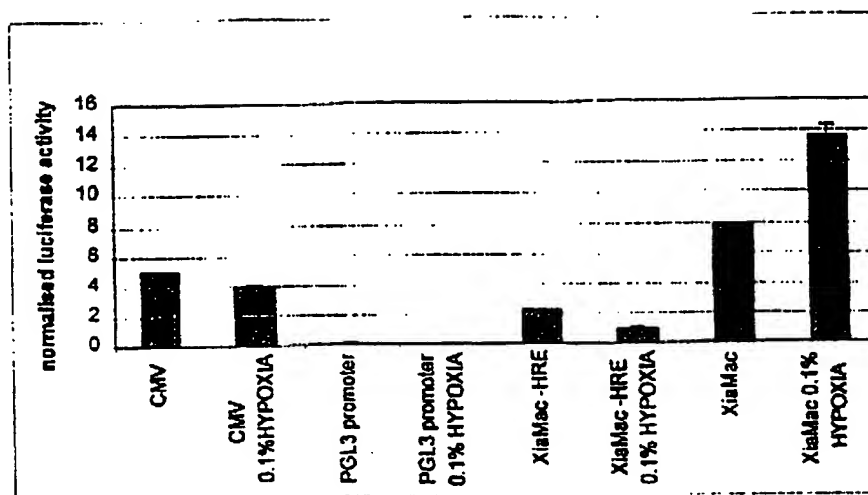


Figure 10

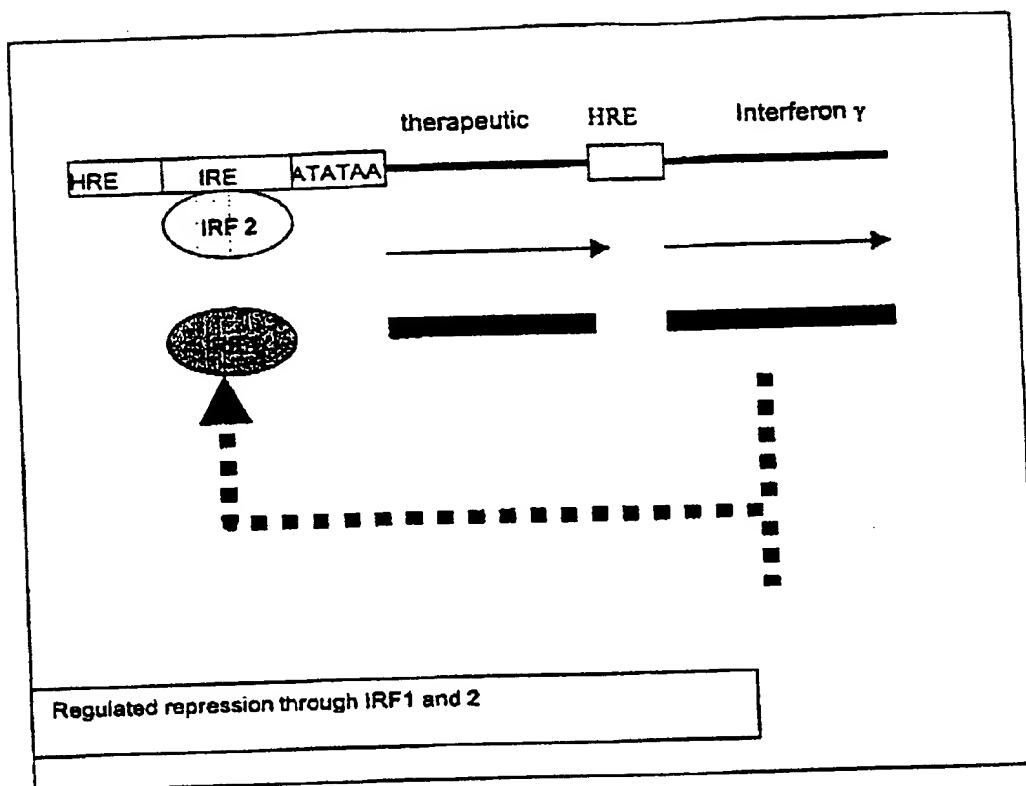


Figure 11

## XiaMac-IRE Sequence

GCTAGAGTCGTGCAGGACGTGACATCTAGTGTCGTGCAGGACGTGACA  
HRE HRE  
TCTAGTGTCGTGCAGGACGTGACAGCTAGCATTCCATCACGTGGCCCG  
HRE NF-IL6  
AGAGAAGCATCCGGAGTACTACAAGGACTGCTGACAGCGAGATTCTA  
CAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGTGTGGCCTGGGCG  
NFkB NFkB SP1 SP1  
GGACTGGGGAGTGGC AAGTGAAAGTGAAAGTGAAAGTGA  
SP1 IRE IRE IRE IRE  
GAGCCCTCAGATGCTGC ~~ATTTT~~ GCAGCTGCTTTTGCCC

Figure 12

# Configuration of HRE regulated endothelial specific lentiviral vectors

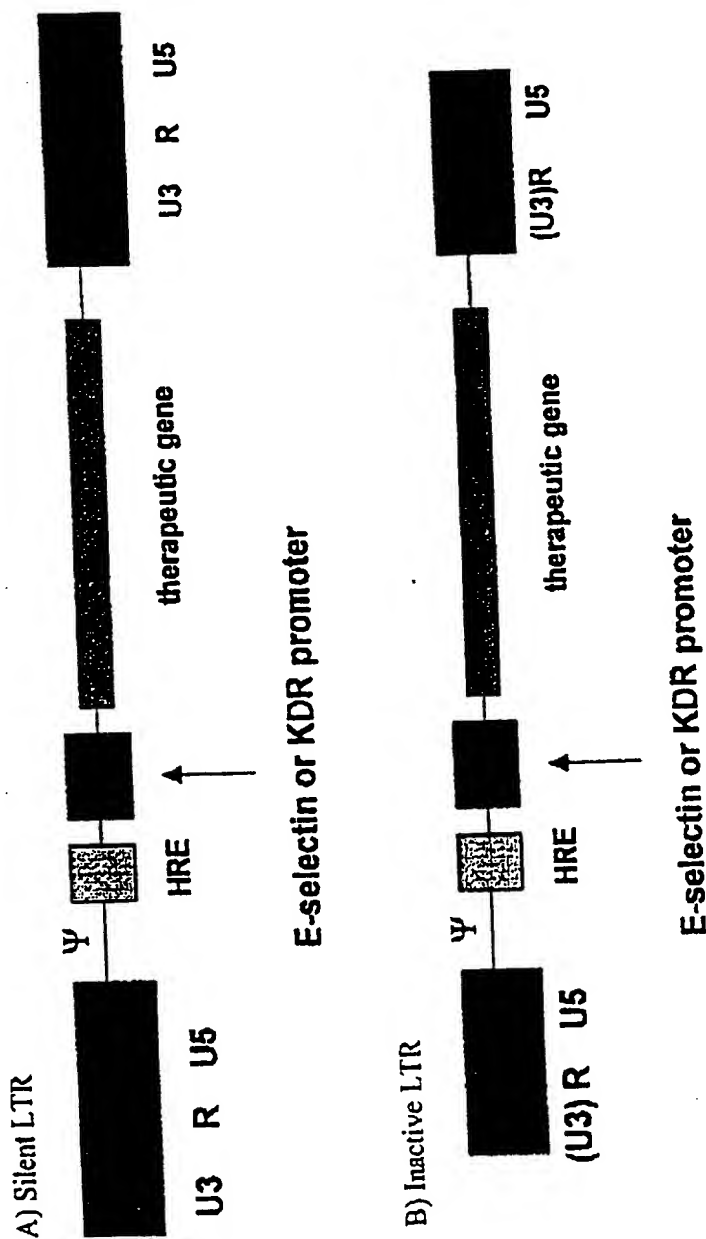


Figure 13

WT PGK18+++

(NheI )  
5' CTA GCT GTC ACG TCC TGC ACG ACA CTA GAT GTC ACG TCC TGC  
GA CAG TGC AGG ACG TGC TGT GAT CTA CAG TGC AGG ACG  
ACG ACA CTA GAT GTC ACG TCC TGC ACG ACT  
TGC TGT GAT CTA CAG TGC AGG ACG TGC TGA GAT C 3'  
( Xba I. )

MUT PGK18+++

(NheI )  
5' CTA GCT GTC CAT TCC TGC ACG ACA CTA GAT GTC CAT TCC TGC  
GA CAG GTA AGG ACG TGC TGT GAT CTA CAG GTA AGG ACG  
ACG ACA CTA GAT GTC CAT TCC TGC ACG ACT  
TGC TGT GAT CTA CAG GTA AGG ACG TGC TGA GAT C 3'  
( XbaI )

Figure 14

5

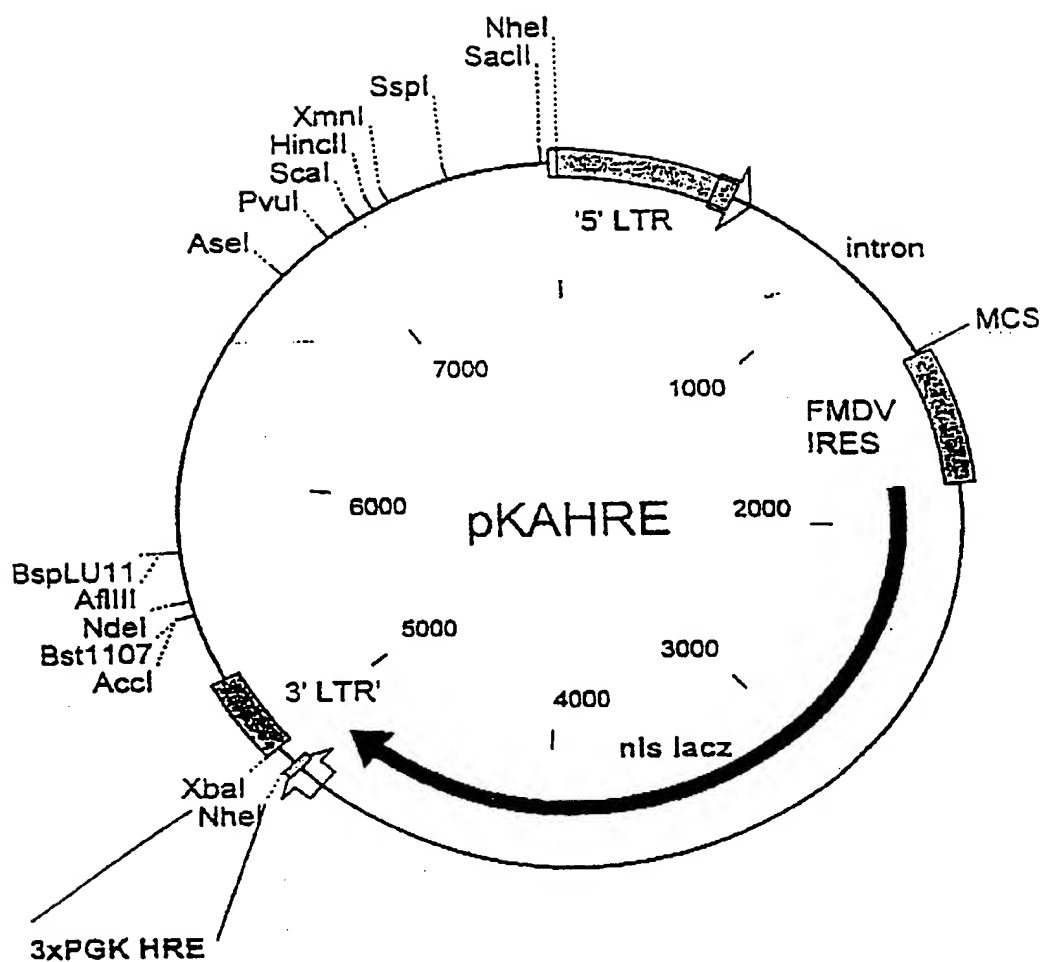
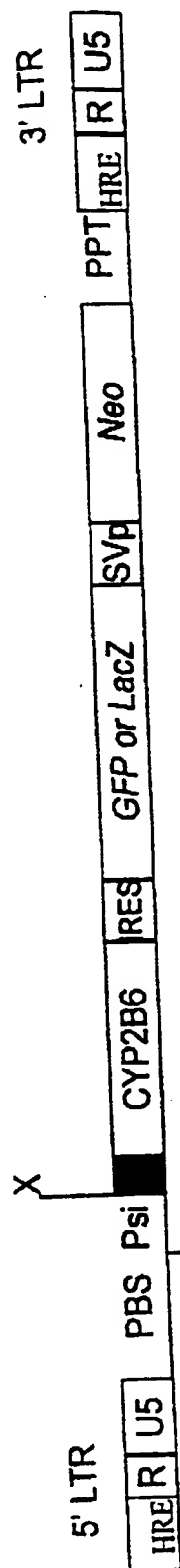




Figure 15a

**XiaGen -P450**

LTR: long terminal repeat

PBS: Primer binding site

Psi: Packaging site

PPT: poly purine tract

IRES: internal ribosome entry site

SVp: SV40 promoter

SD: defective splice donor

Figure 15 b

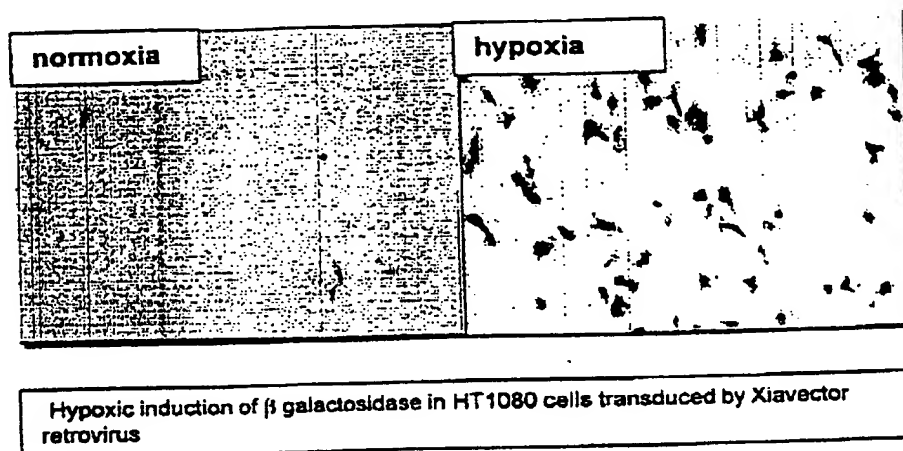


Figure 16a

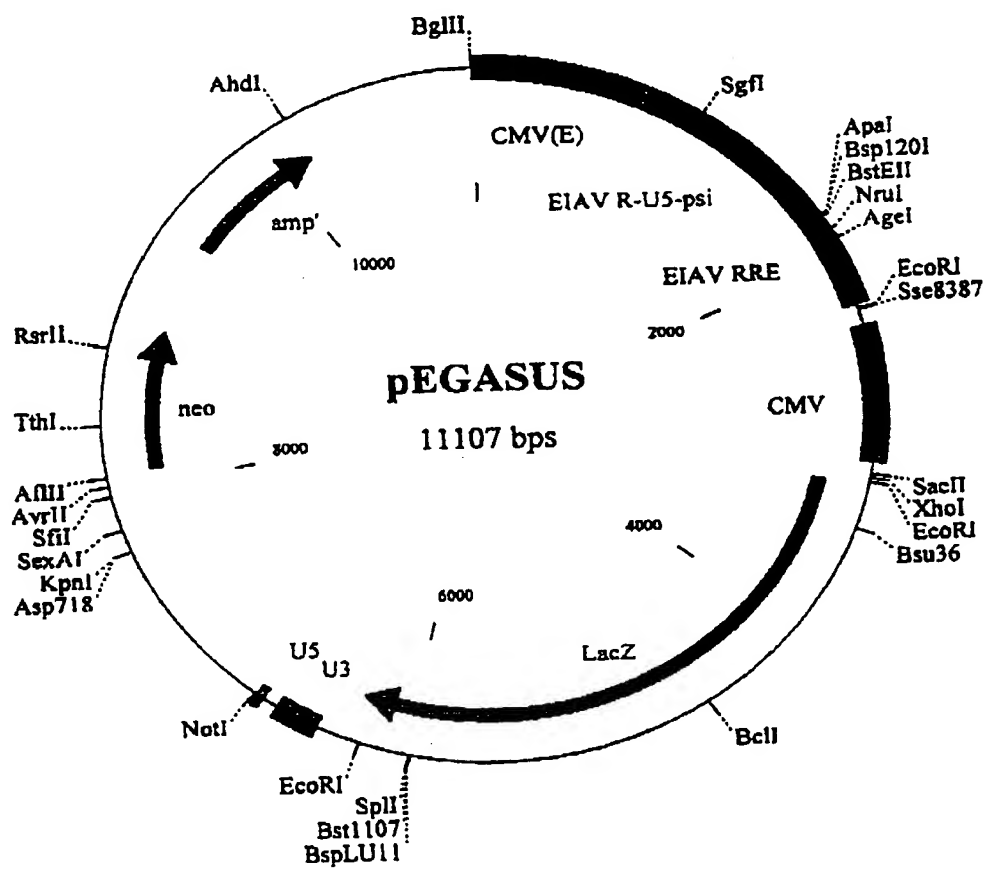


Figure 16b

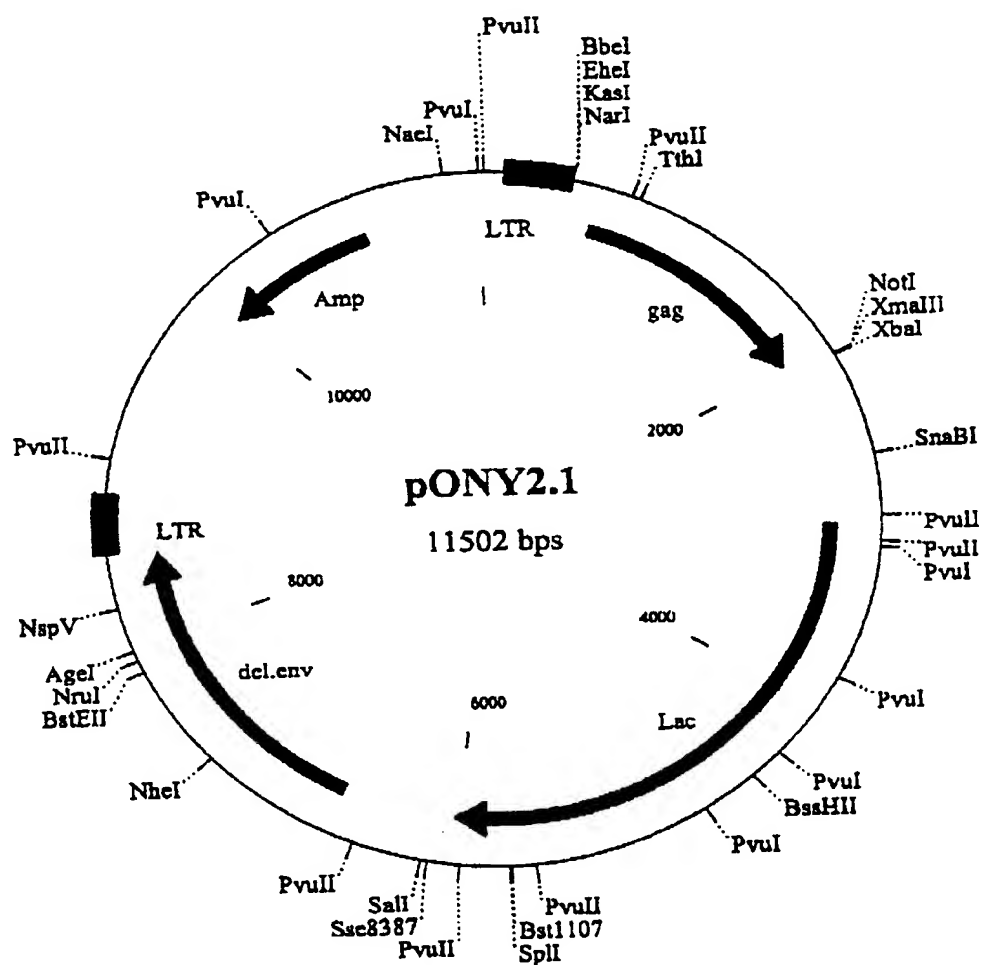


Figure 16c

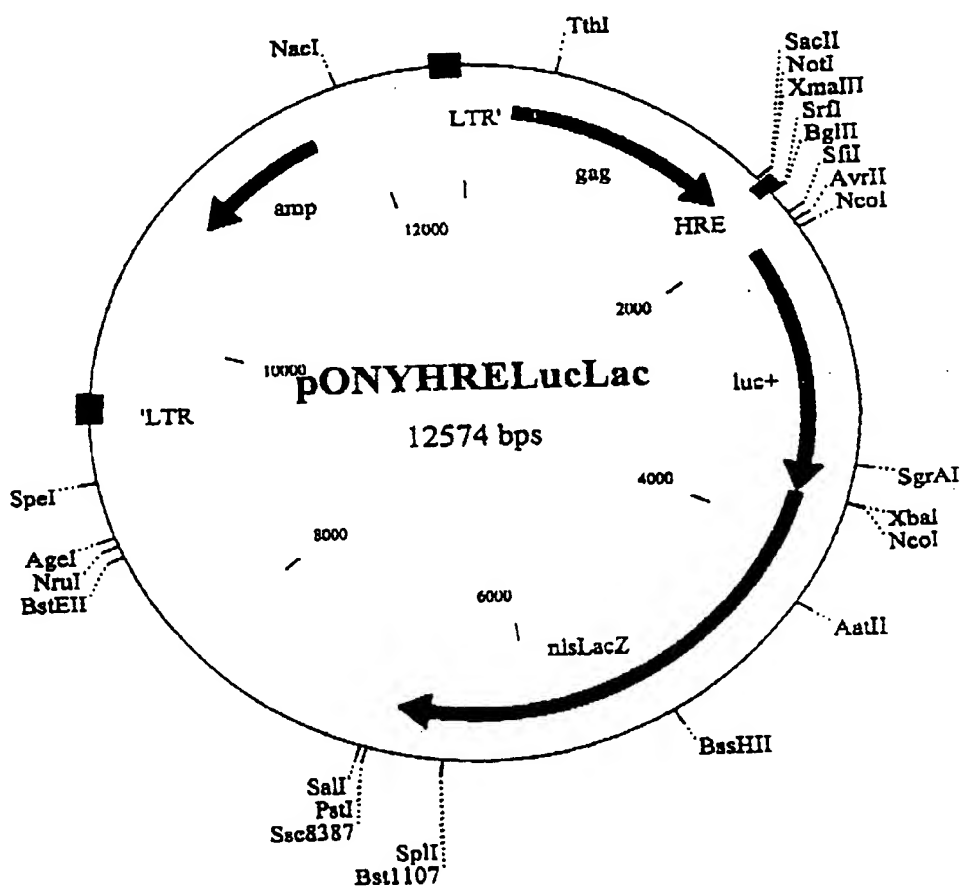


Figure 16d

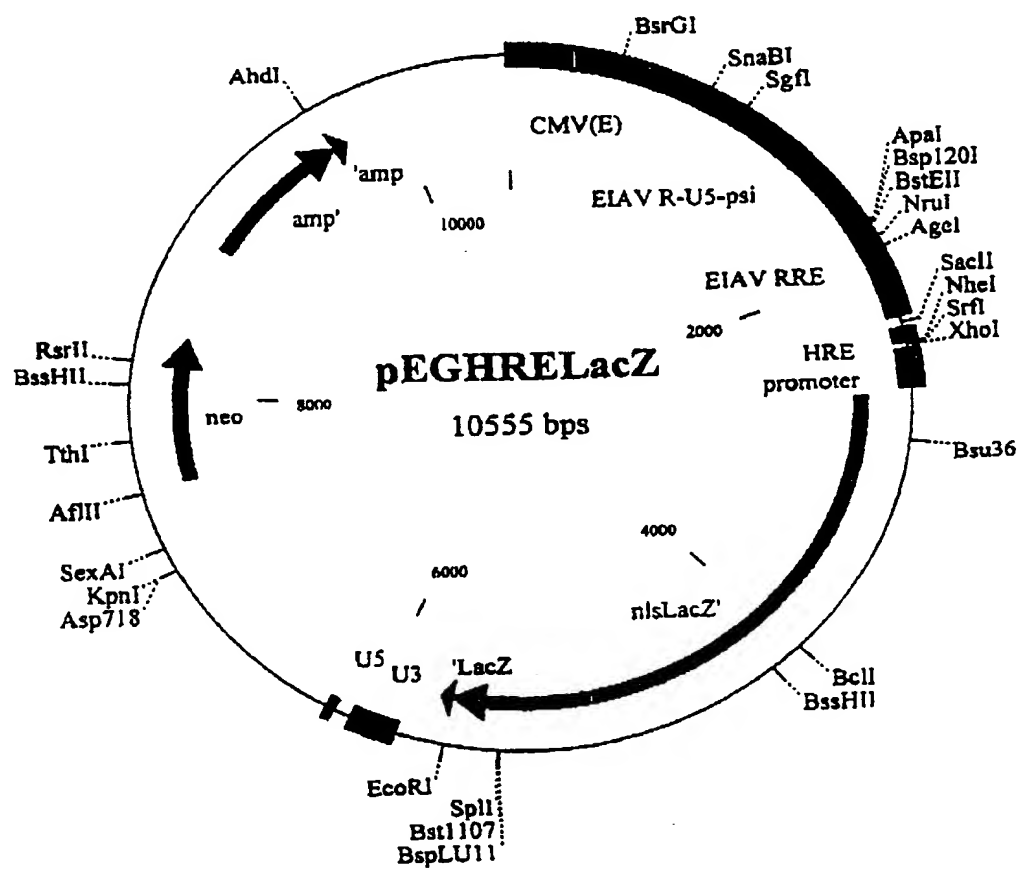


Figure 18

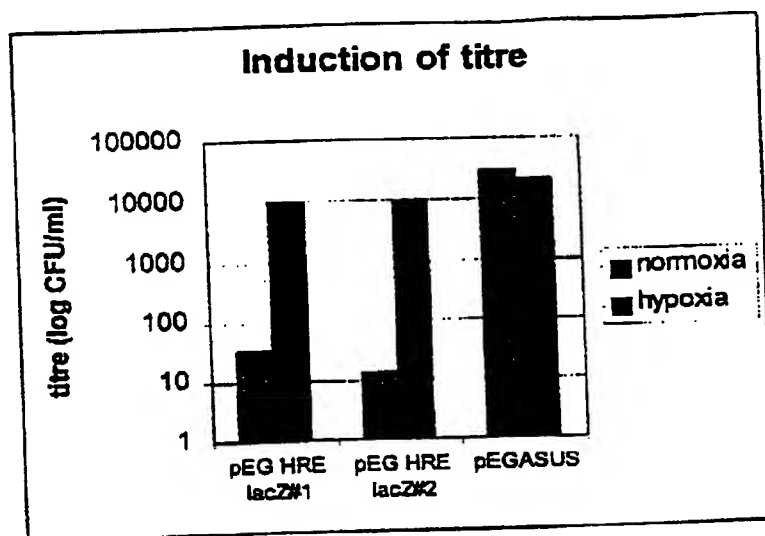


Figure 19

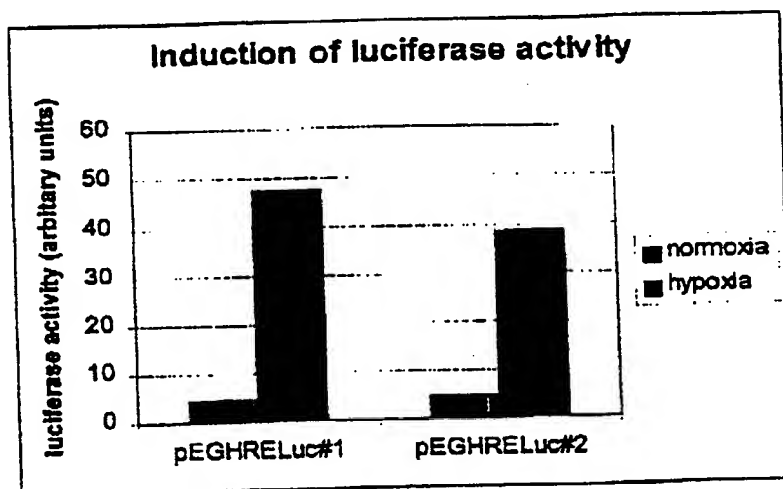
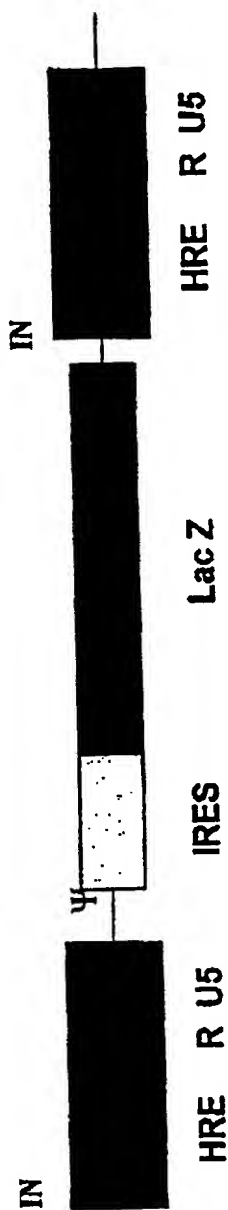




Figure 20A

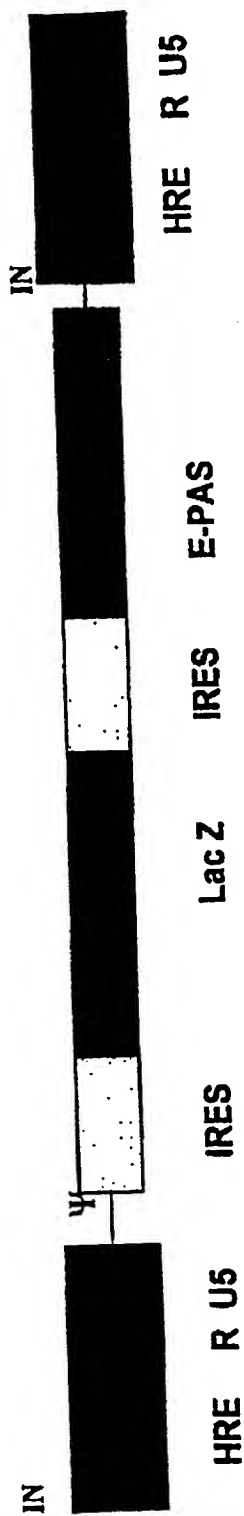
# Hypoxia responsive EIAV vector configured as a single transcription unit



IN = Site for integration via the EIAV integrase  
HRE = Hypoxia response element  
IRES = Internal ribosome entry site  
R = Repeat required for reverse transcription  
U5= part of the EIAV LTR  
ψ = packaging site

Figure 20B

# Hypoxia responsive Autoregulated EIAV vector configured as a single transcription unit



IN = Site for integration via the EIAV integrase

HRE = Hypoxia response element

IRES = Internal ribosome entry site

R = Repeat required for reverse transcription

U5= part of the EIAV LTR

ψ = packaging site

E-PAS = endothelial PAS gene

Figure 21

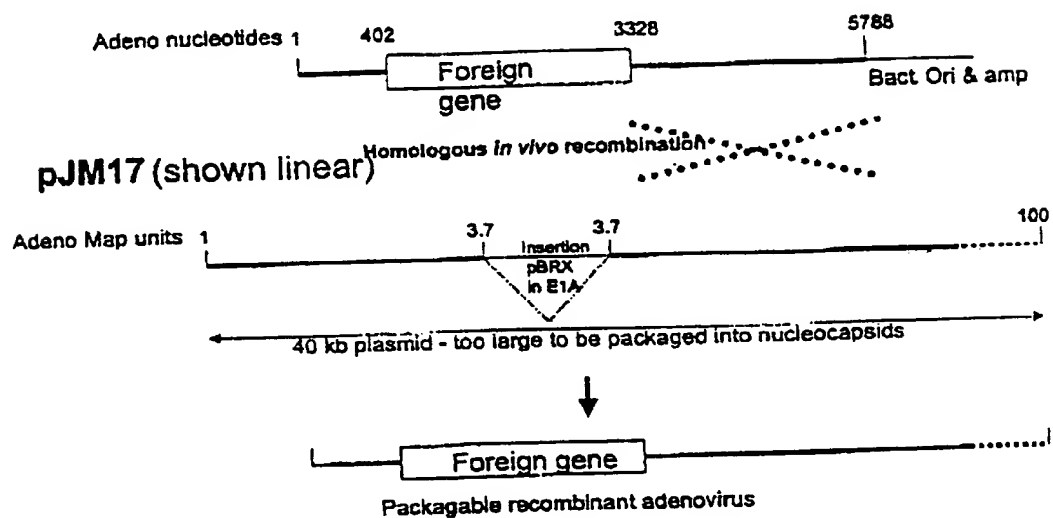
**Transfer vector- pE1sp1A (shown linear)**

Figure 22

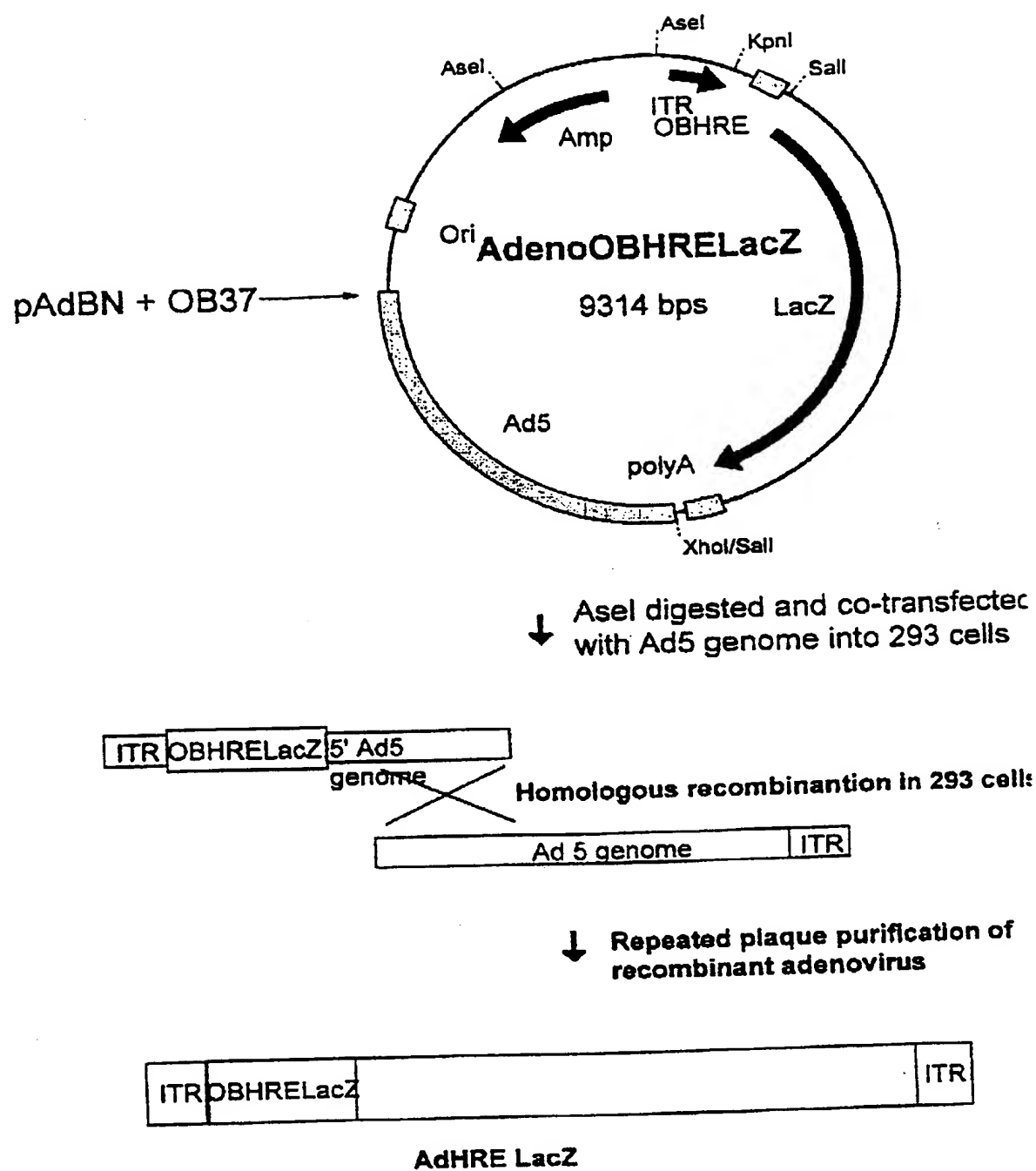
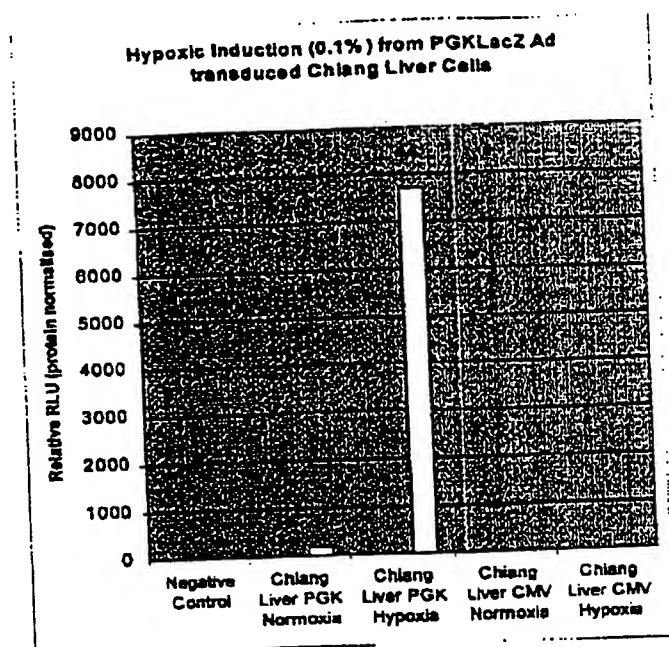


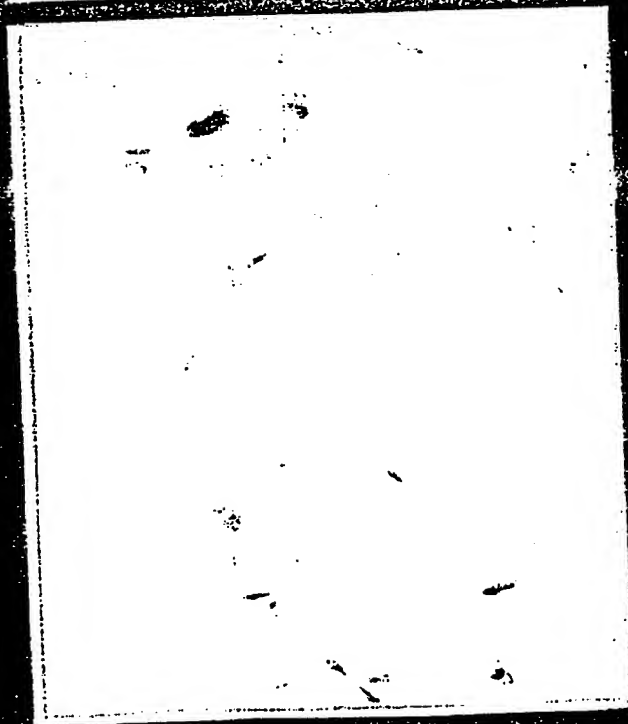
Figure 23



Hypoxic regulation of lacZ in primary human macrophages transduced with OB HRE1 Adenovirus



hypoxia



normoxia

Figure 24

Figure 25a

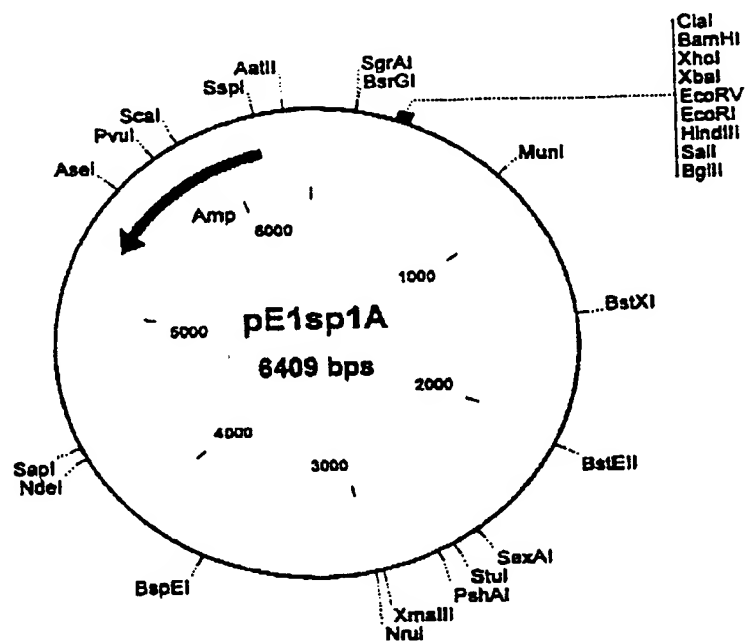


Figure 25b

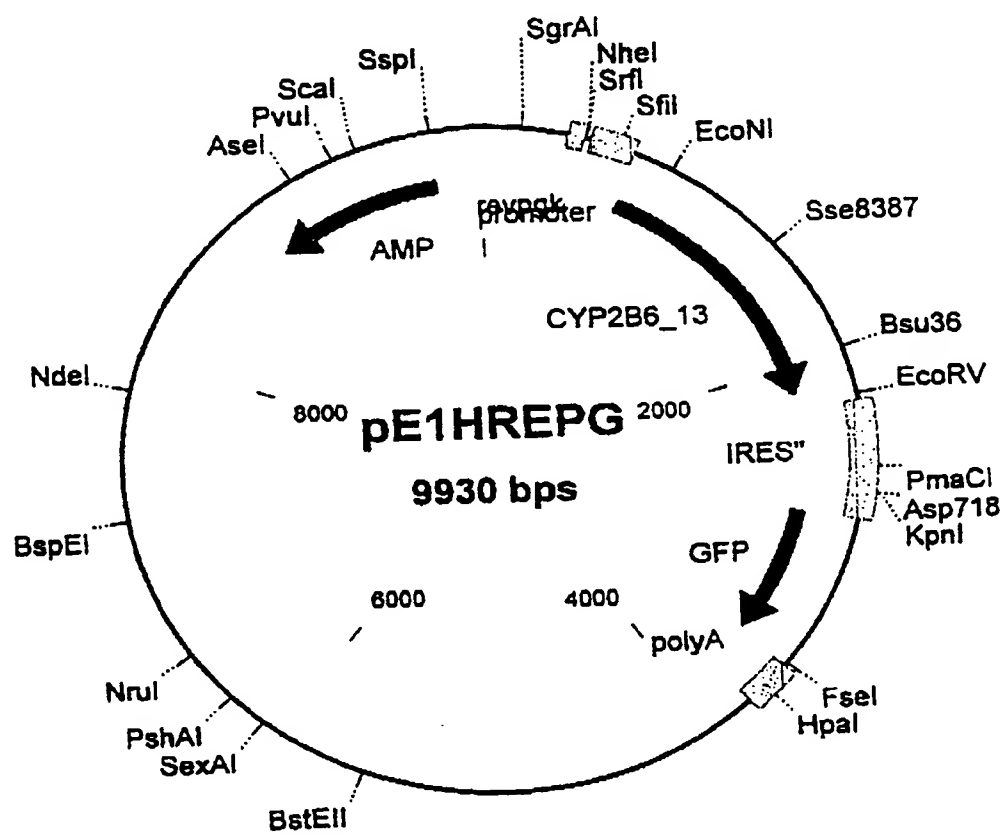




Figure 25c

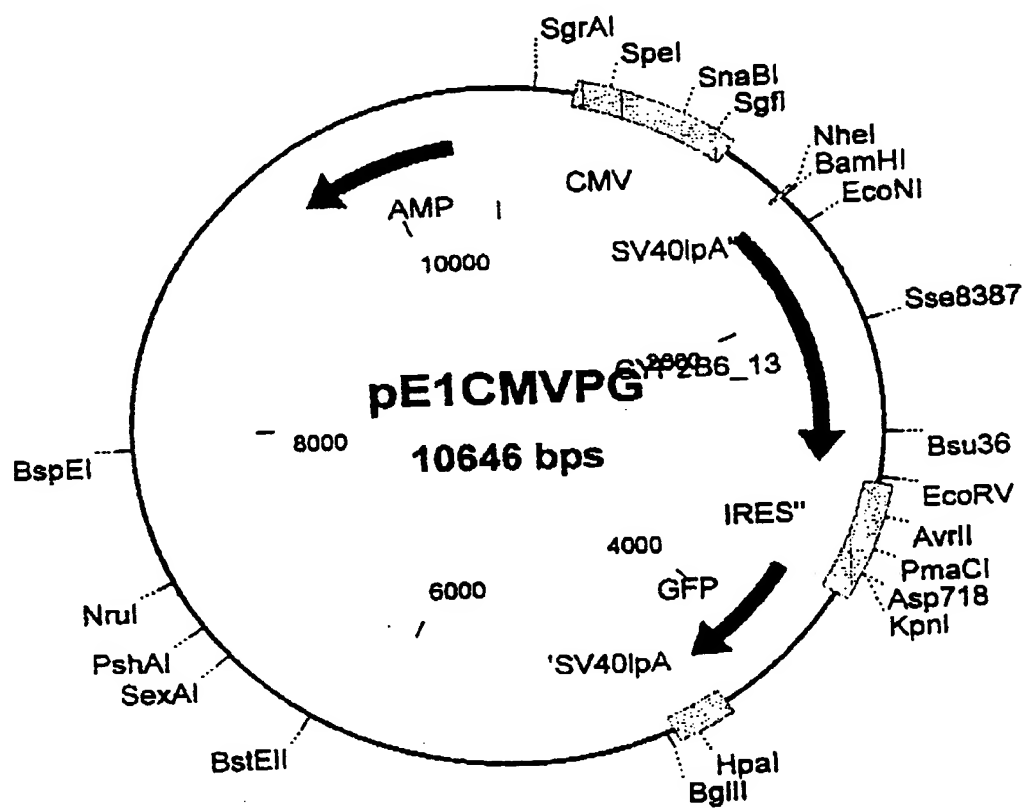


Figure 26

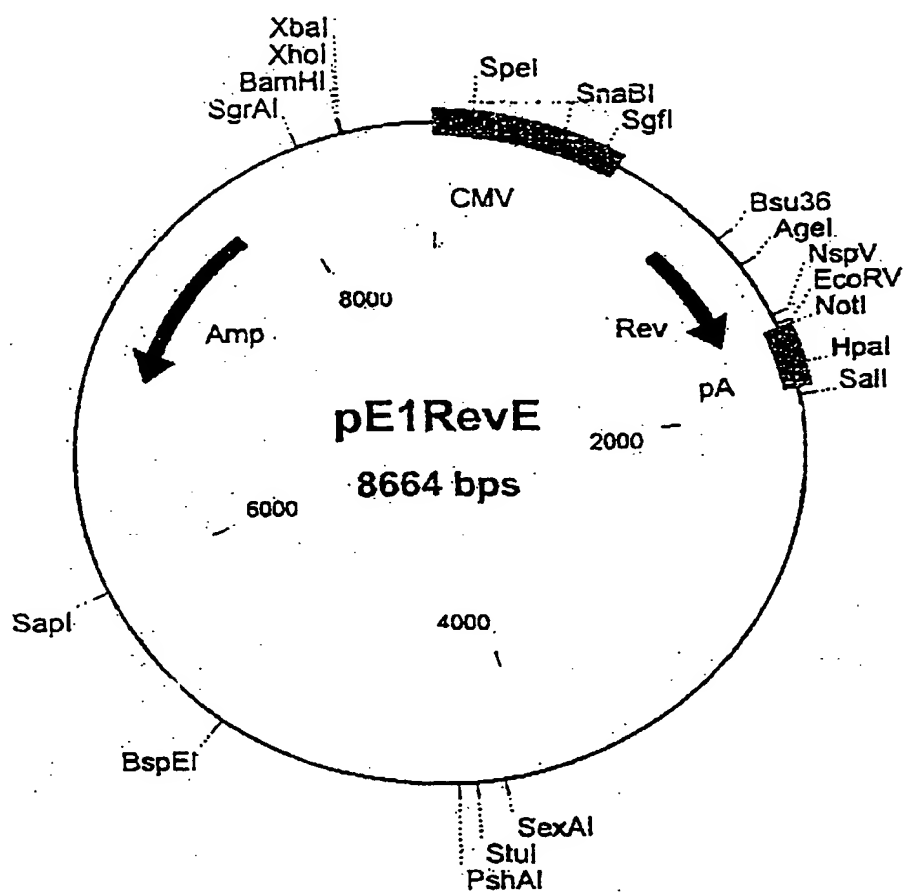


Figure 27

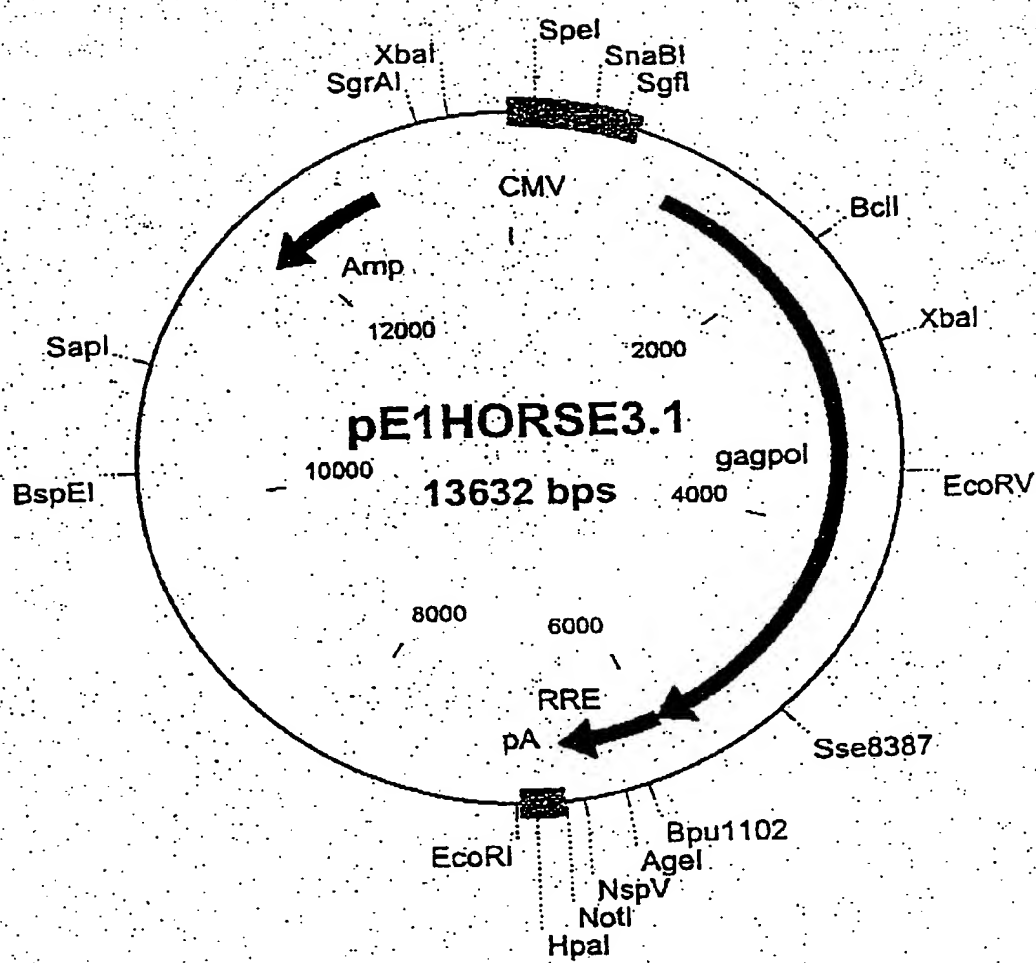


Figure 28

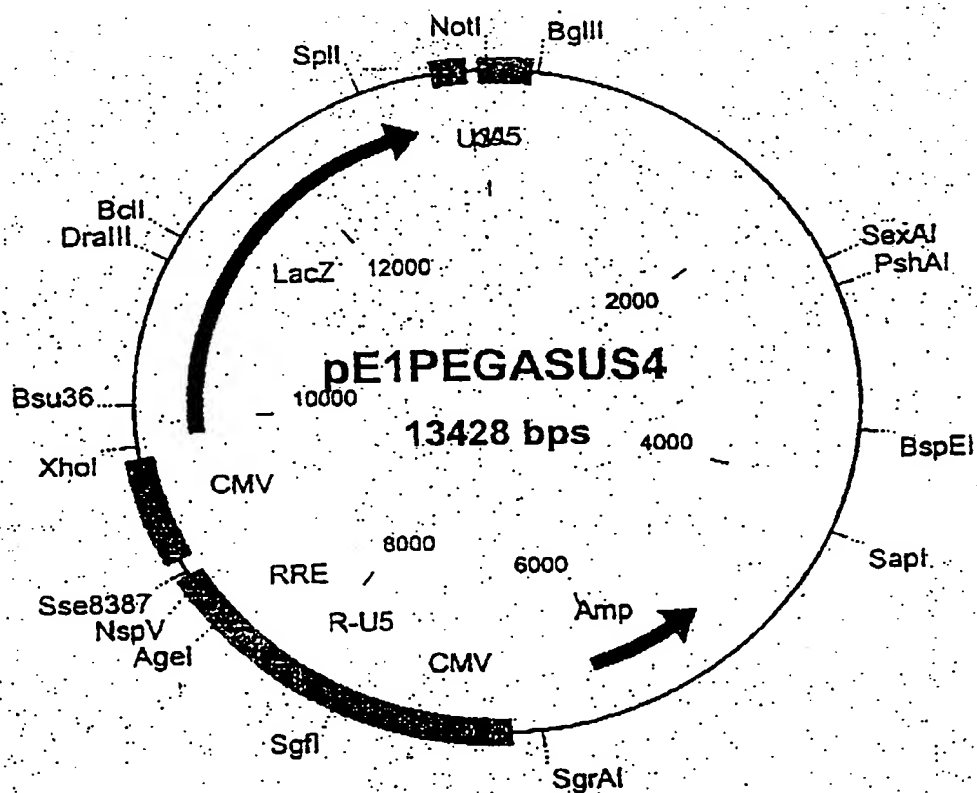


Figure 29

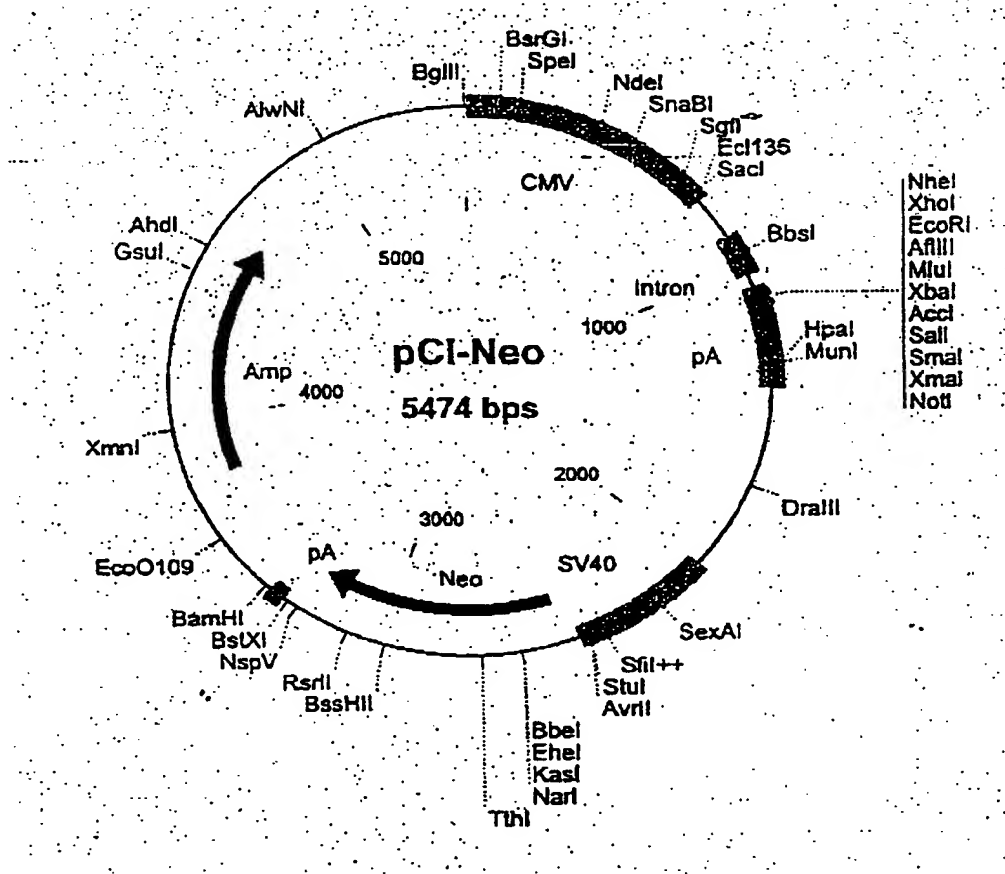
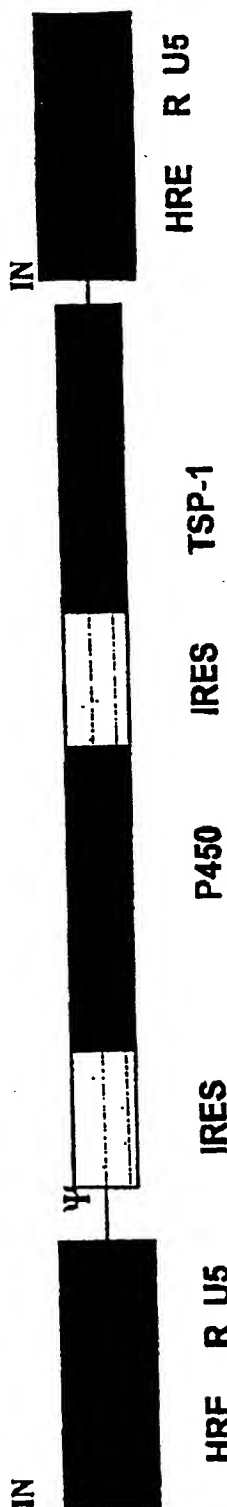


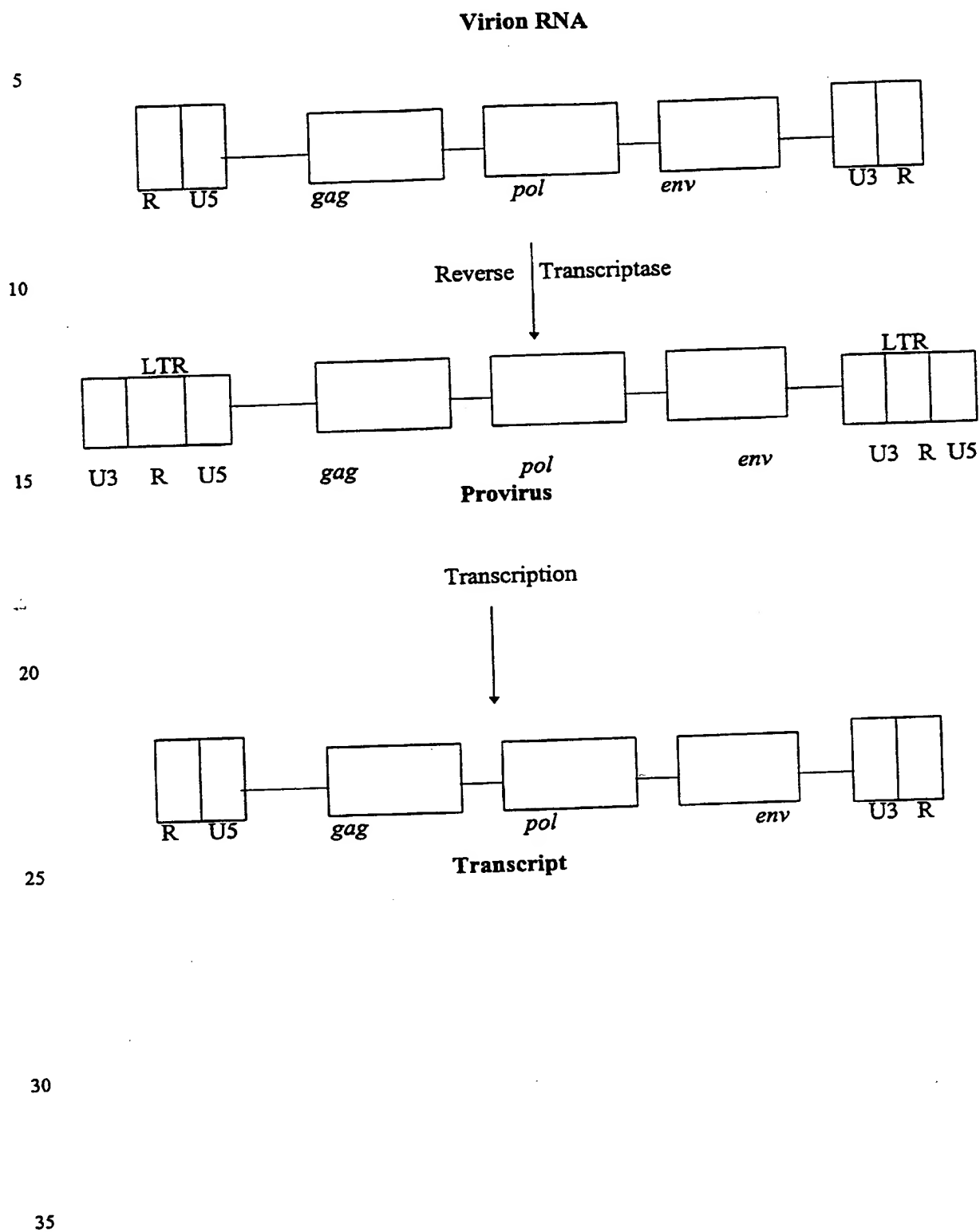
Figure 32

# Hypoxia responsive EIAV vector containing two therapeutic genes



IN = Site for integration via the EIAV integrase  
HRE = Hypoxia response element  
IRES = Internal ribosome entry site  
R = Repeat required for reverse transcription  
U5 = part of the EIAV LTR  
ψ = packaging site  
P450 = Human CYP2B6 gene  
TSP-1 = Human thrombospondin-1

Figure 33



## ANNEX

Figure 1:

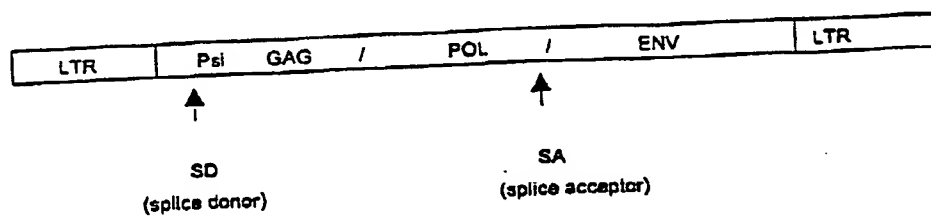




Figure 2.

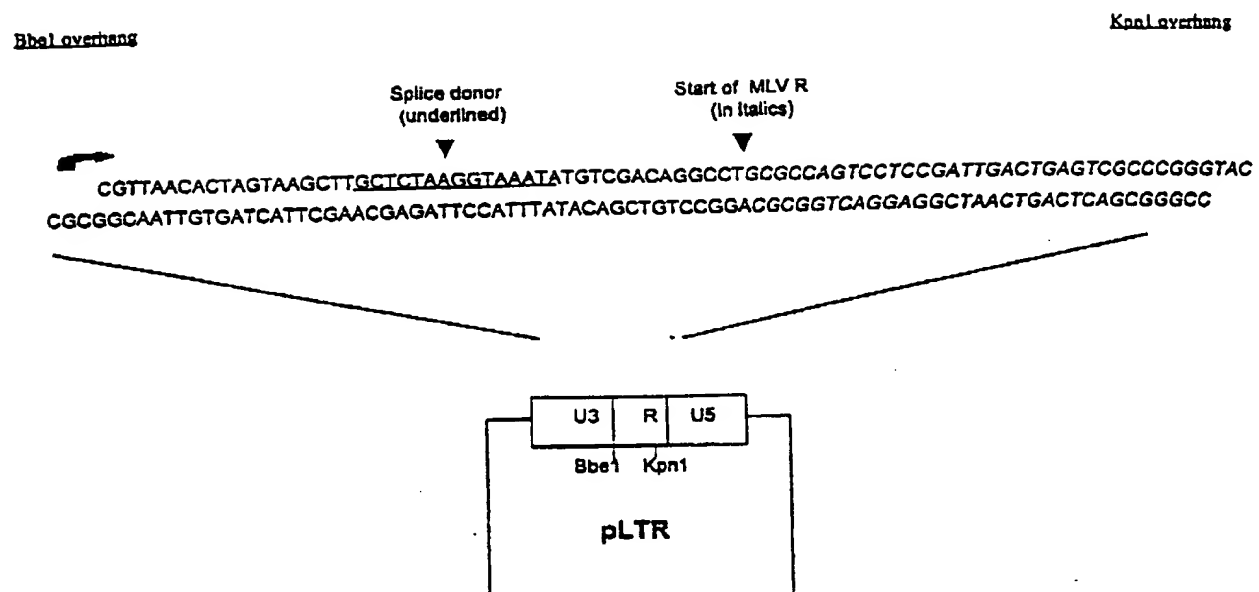


Figure 3:

BamH1 compatible

5' gatctaacctaggtcttcgagtgcttaaacactgggcttgcgagacagagaagactcttgcgtttctgatataggcacctatttggtcttactgac-  
 3' attggatccagagctcacaaatttgtagcccgaaacagctctgtctcttctgagaacgcaaaagactatccgtggataaccagaatgactg-

SmaI compatible

SA

-atccactttggtttctctctccacacagtgagg-3'  
 -taggtgaacgggaaagagaggtgtccactcc-5'

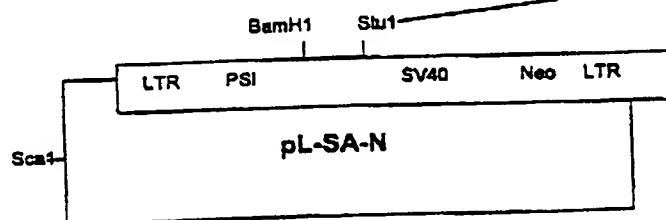


Figure 4:

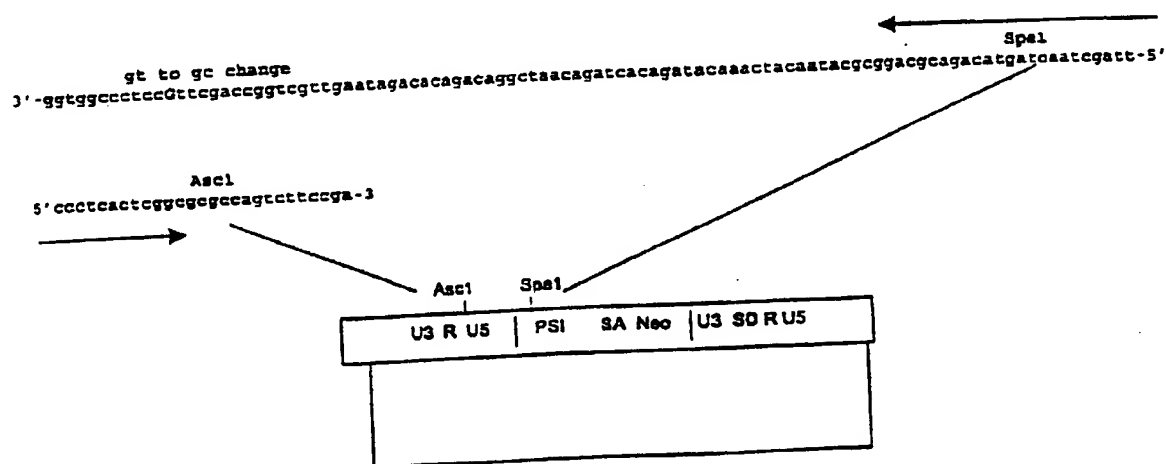


Figure 5

GCTAGCTTAAGTAACGCCACTTTGCAAGGCATGGAAAAATACATAACTGAGAATAGAAAAAGTTCA  
GATCAAGGTCAGGAACAAAGAAACAGCTGAATACCAAACAGGATATCTGTGGTAAGCGGTTCTCTG  
5 CCCCCGCTCAGGGCCAAGAACAGATGAGACAGCTGAGTGATGGGCCAAACAGGATATCTGTGGTA  
AGCAGTTCTCTGCCCGGCTCGGGGCCAAGAACAGATGGTCCCCAGATGCGGTCCAGCCCTCAGCA  
GTTTCTAGTGAATCATCAGATGTTTCCAGGGTGCCCCAAGGACCTGAAAATGACCCTGTACCTTAT  
TTGAACTAACCAATCAGTTCGCTTCTCGCTTCTGTTCCGCGCTTCCGCTCTCCGAGCTCAATAAA  
AGAGCCCAACCCCTCACTCGGCGCGCCAGTCTTCCGATAGACTGCGTCGCCCCGGGTACCCGTA  
10 TTCCCAATAAAGCCTCTTGCTGTTTGCATCCGAATCGTGGTCTCGCTGTTCTTGGGAGGGTCTCCT  
CTGAGTGATTGACTACCCACGACGGGGGTCTTTCAATTTGGGGGCTCGTCCGGGATTGGAGACCCC  
TGCCAGGGACACCGACCCACCACCGGGAGGCAAGCTGGCCAGCAACTTATCTGTGTCTGTCCG  
ATTGCTAGTGTCTATGTTTGATGTTATGCGCCTGCGTCTGTACTAGTTAGCTAACTAGCTCTGTAT  
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Figure 6.

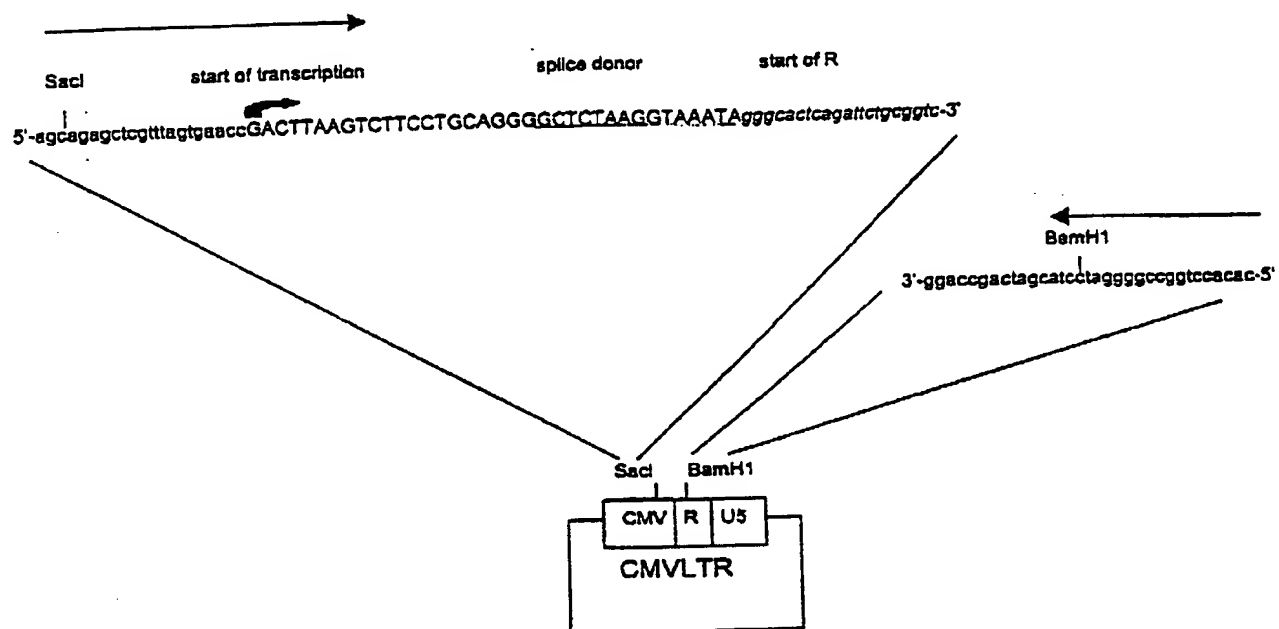


Figure 7

Xho1  
overhang

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3'-gcaaatltgtgacccgaacagctctgtctcttctgagaacgcaaagactatccgtggataaccagaatgactgtagggtgaaacgg-

SA

Bpu1102 overhang

-tttctctccacaggtcacgtgaagctagcctcgagtggc-3'  
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Xho1

Bpu1102

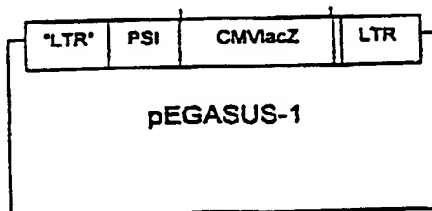


Figure 8

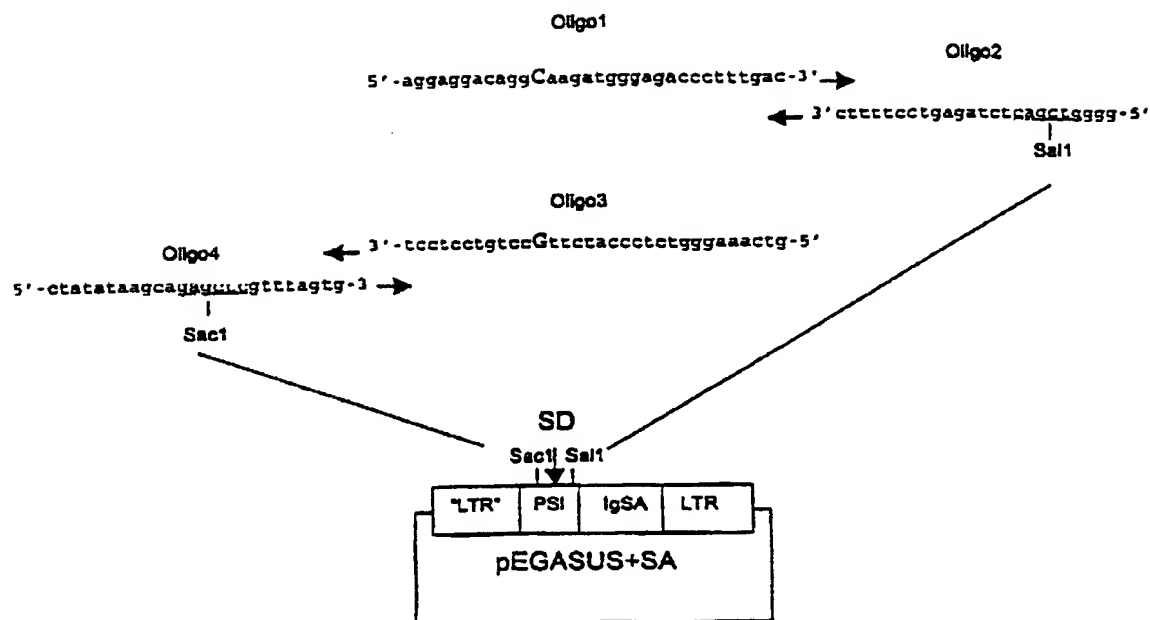




Figure 9

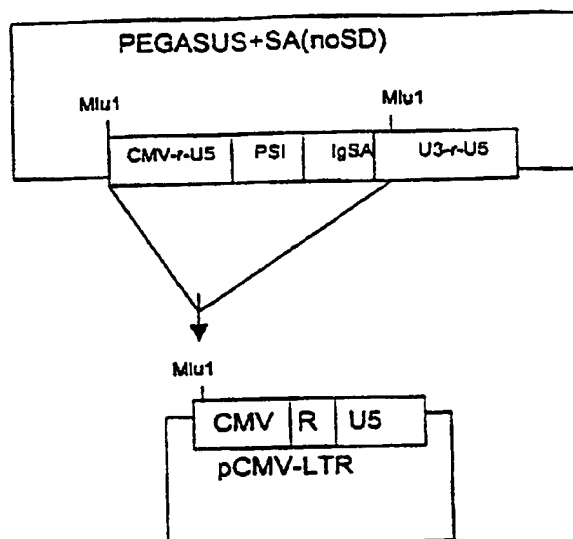


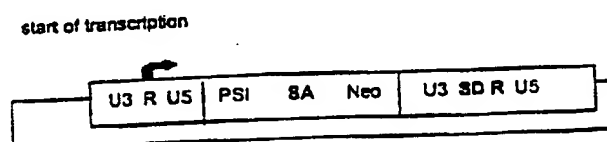
Figure 11

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*Figure 12:*

(A) pICUT vector in transfected cells



(B) pICUT vector in transduced cells

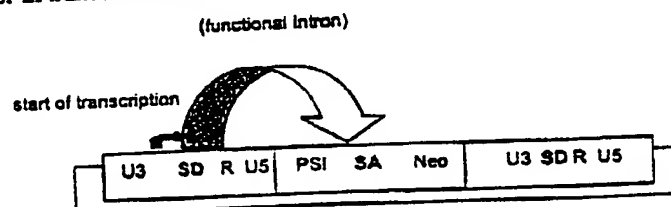
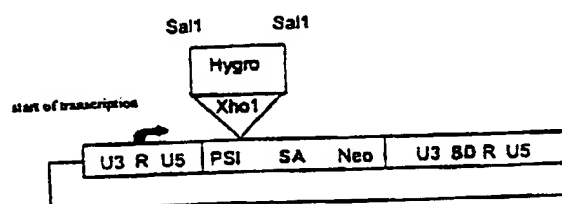


Figure 13:

(a) Vector configuration in transfected cells



(b) Vector configuration in transduced cells

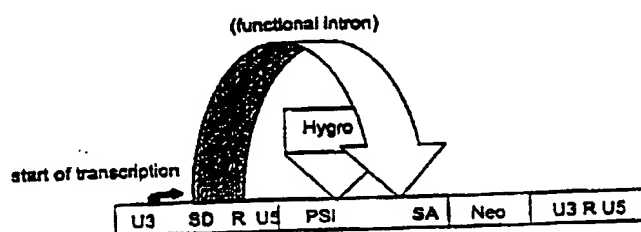
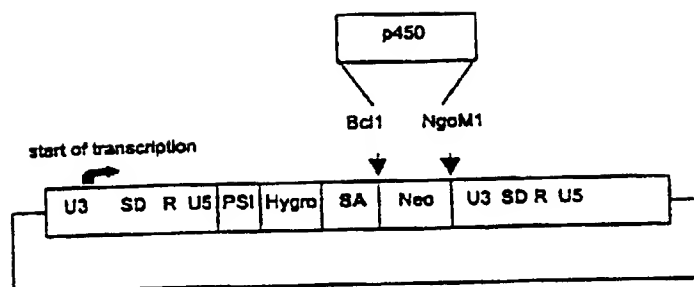


Figure 14

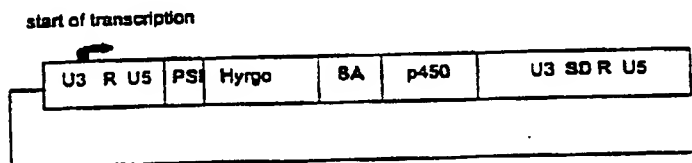
## (A) p450 insertion

p450 cDNA is inserted into the Bcl1/NgoM1 site to replace the Neo gene



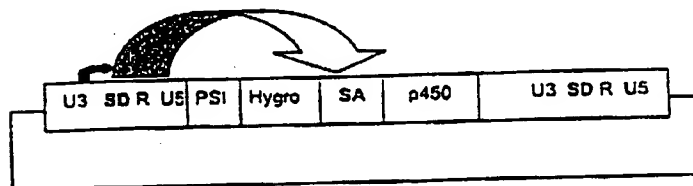
## (B) Transfected cells

The hygromycin gene is the first ORF in the transfected cell and thus expressed from the transcripts. Without any IRES, p450 translation will not occur



## (C) Transduced Cells

The hygromycin gene is now contained within an active intron and thus removed from transcripts thus allowing expression of p450



*Figure 15:*

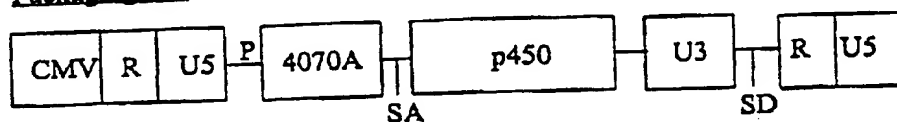
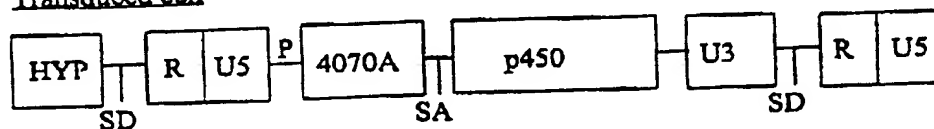
3'end of pol	5'-ATG CGT TCA ACG CTC TCA AAA CCC CTT AAA AAT AAG
5'altered 4070A	5'-ATG GCC AGA AGC ACC CTG AGC AAG CCA CCC CAG GAC
	GTT AAC CCG CGA GGC CCC CTA ATC CCC-3'
	AAA AAT CCC TGG AAA CCT CTG ATC GTC-3'

Figure 16:

ATGGCCAGAA GCACCCTGAG CAAGCCACCC CAGGACAAAA TCAATCCCTG GAAACCTCTG  
ATCGTCATGG GAGTCCTGTT AGGAGTAGGG ATGGCAGAGA GCCCCCATC AGGTC  
TTTAATGTAA CCTGGAGAGT CACCAACCTG  
ATGACTGGGC GTACCGCCAA TGCCACCTCC CTCCTGGGAA CTGTACAAGA TGCCTTCCCA  
AAATTATATT TTGATCTATG TGATCTGGTC GGAGAGGAGT GGGACCCTTC AGACCAGGAA  
CCGTATGTCC GGTATGGCTG CAAGTACCCC GCAGGGAGAC AGCGGACCCG GACTTTTQAC  
TTTTACGTGT GCCCTGGGCA TACCGTAAAG TCGGGGTGTG GGGGACCAGG AGAGGGCTAC  
TGTGGTAAAT GGGGGTGTGA AACCACCGGA CAGGCTTACT GGAAGCCAC ATCATCGTGG  
GACCTAATCT CCCTTAAGCG CGGTAACACC CCCTGGGACA CGGGATGCTC TAAAGTTGCC  
TGTGGCCCCCT GCTACGACCT CTCAAAGTA TCCAATTCCT TCCAAGGGGC TACTCGAGGG  
GGCAGATGCA ACCCTCTAGT CCTAGAATTC ACTGATGCAG GAAAAAAGGC TAACTGGGAC  
GGGCCCCAAT CGTGGGGACT GAGACTGTAC CGGACAGGAA CAGATCCTAT TACCATGTTT  
TCCCTGACCC GGCAGGTCCT TAATGTGGGA CCCCAGTCC CCATAGGGCC CAACCCAGTA  
TTACCCGACC AAAGACTCCC TTCCTCACCA ATAGAGATTG TACCGGCTCC ACAGCCACCT  
AGCCCCCTCA ATACCAGTTA CCCCCCTTCC ACTACCAGTA CACCCTCAAC CTCCCCTACA  
AGTCCAAGTG TCCCACAGCC ACCCCCAGGA ACTGGAGATA GACTACTAGC TCTAGTCAAA  
GGAGCCTATC AGGCGCTTAA CCTCACCAAT CCCGACAAGA CCCAAGAATG TTGGCTGTGC  
TTAGTGTGCG GACCTCCTTA TTACGAAGGA GTAGCGGTCC TGGGCACTTA TACCAATCAT  
TCCACCGCTC CGGCCAACTG TACGGCCACT TCCCAACATA AGCTTACCCT ATCTGAAGTG  
ACAGGACAGG GCCTATGCAT GGGGGCAGTA CCTAAAACTC ACCAGGCCTT ATGTAACACC  
ACCCAAAGCG CCGGCTCAGG ATCCTACTAC CTTGCAGCAC CCGCCGGAAC AATGTGGGCT  
TGCAGCACTG GATTGACTCC CTGCTTGTCC ACCACGGTGC TCAATCTAAC CACAGATTAT  
TGTGTATTAG TTGAACTCTG GCCCAGAGTA ATTTACCACT CCCCAGATTA TATGTATGGT  
CAGCTTGAAC AGCGTACCAA ATATAAAAGA GAGCCAGTAT CATTGACCCT GGCCCTTCTA  
CTAGGAGGAT TAACCATGGG AGGGATTGCA GCTGGAATAG GGACGGGGAC CACTGCCTTA  
ATTAACCC AGCAGTTTGA GCAGCTTCAT GCCGCTATCC AGACAGACCT CAACGAAGTC  
GAAAAGTCAA TTACCAACCT AGAAAAGTCA CTGACCTCGT TGTCTGAAGT AGTCCTACAG  
AACCGCAGAG GCCTAGATTT GCTATTCCTA AAGGAGGGAG GTCTCTGCGC AGCCCTAAAA  
GAAGAATGTT GTTTTTATGC AGACCACAGG GGGCTAGTGA GAGACAGCAT GGCCAAATTA  
AGAGAAAGGC TTAATCAGAG ACAAAAATA TTTGAGACAG GCCAAGGATG GTTCGAAGGG  
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GTACTCTTAC TGATCTTACT CTTTGGACCT TGCATTCTCA ATCGATTGGT CCAATTTGTT  
AAAGACAGGA TCTCAGTGGT CCAGGCTCTG GTTTTGACTC AGCAATATC CCAGCTAAAA  
CCCATAGAGT ACGAGCCATG A



Figure 17:

Packaging cellTransduced cell

- |       |                                |
|-------|--------------------------------|
| CMV   | = CMV Promoter                 |
| HYP   | = Hypoxia responsive promoter  |
| P     | = MLV packaging signal         |
| 4070A | = MLV amphotrophic Env gene    |
| p450  | = p450 reductase encoding cDNA |
| SD    | = Splice donor                 |
| SA    | = Splice acceptor              |

Figure 18:

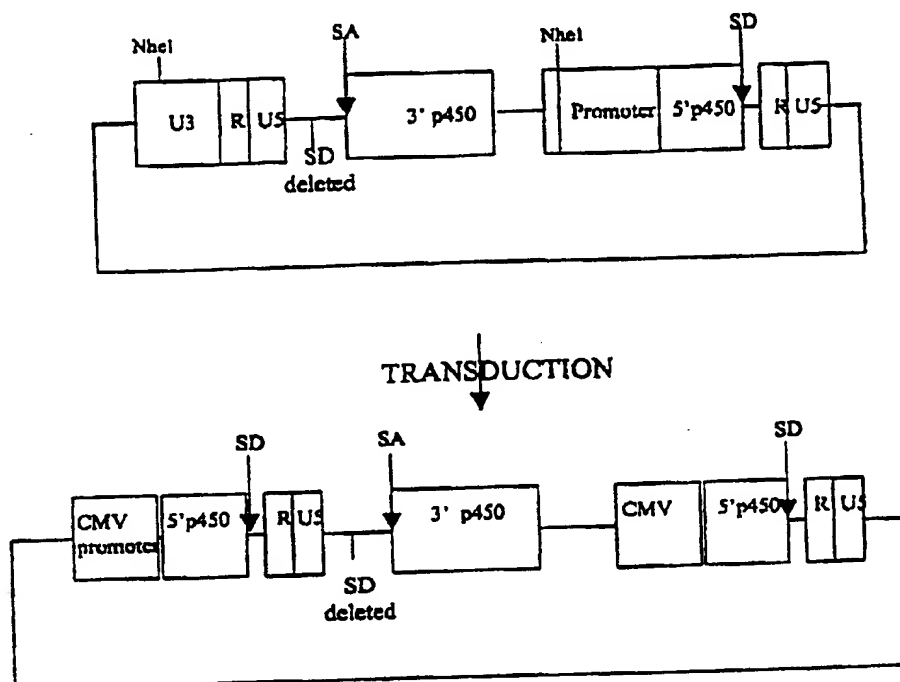


Figure 19

## Transfer vector- pE1sp1A (shown linear)

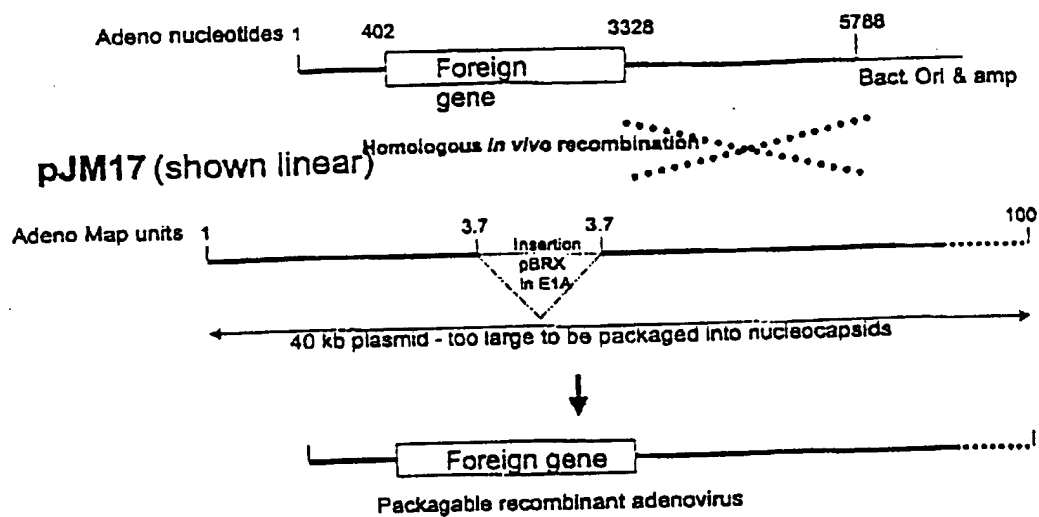


Figure 20

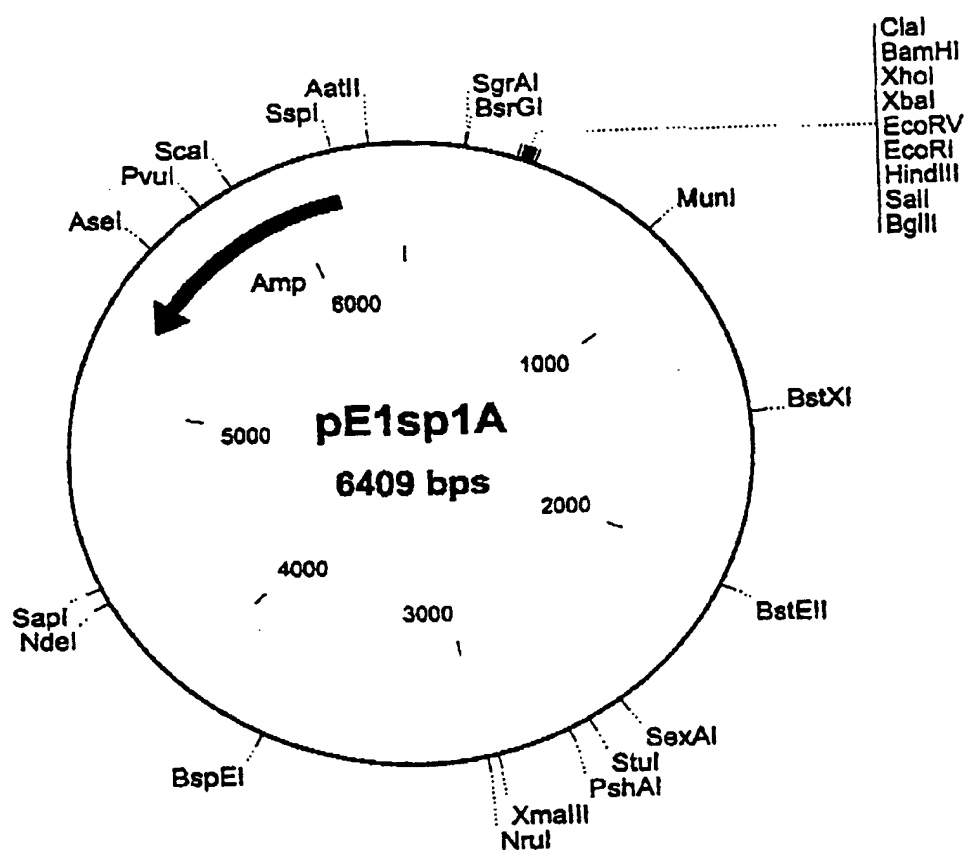


Fig 21

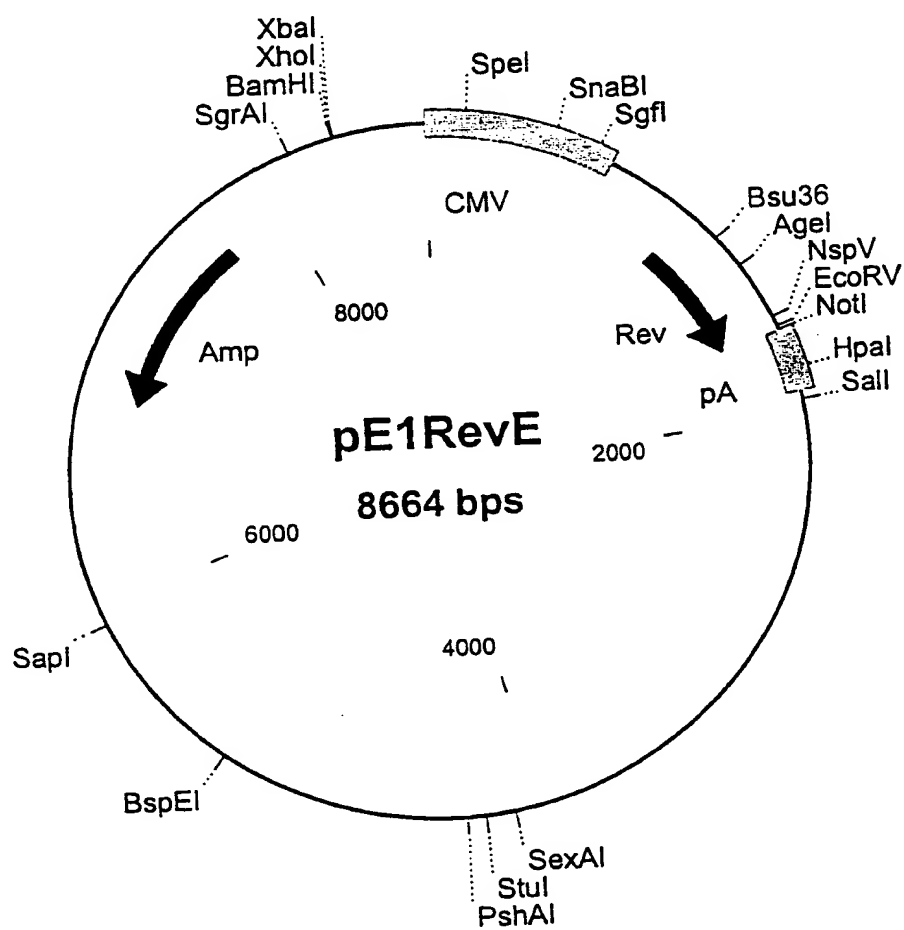


Fig 22

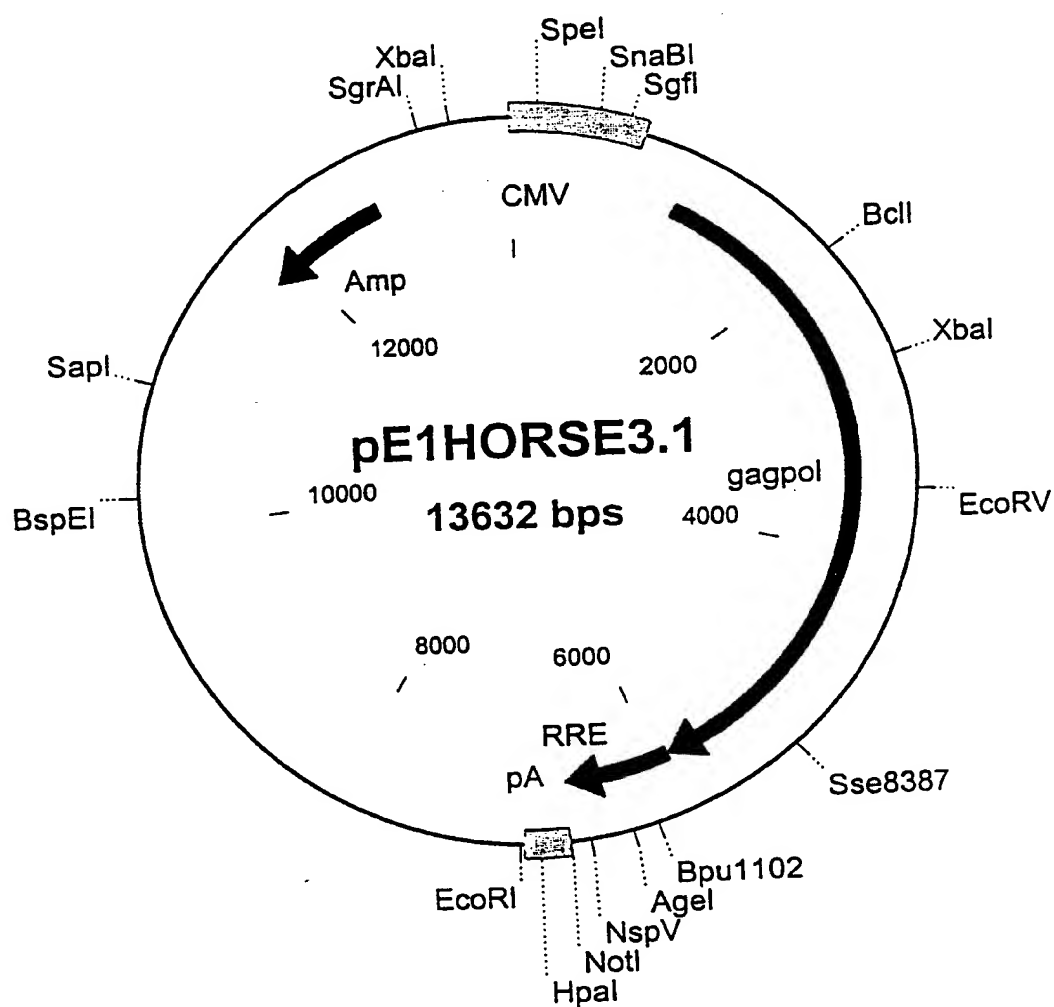




Fig 24

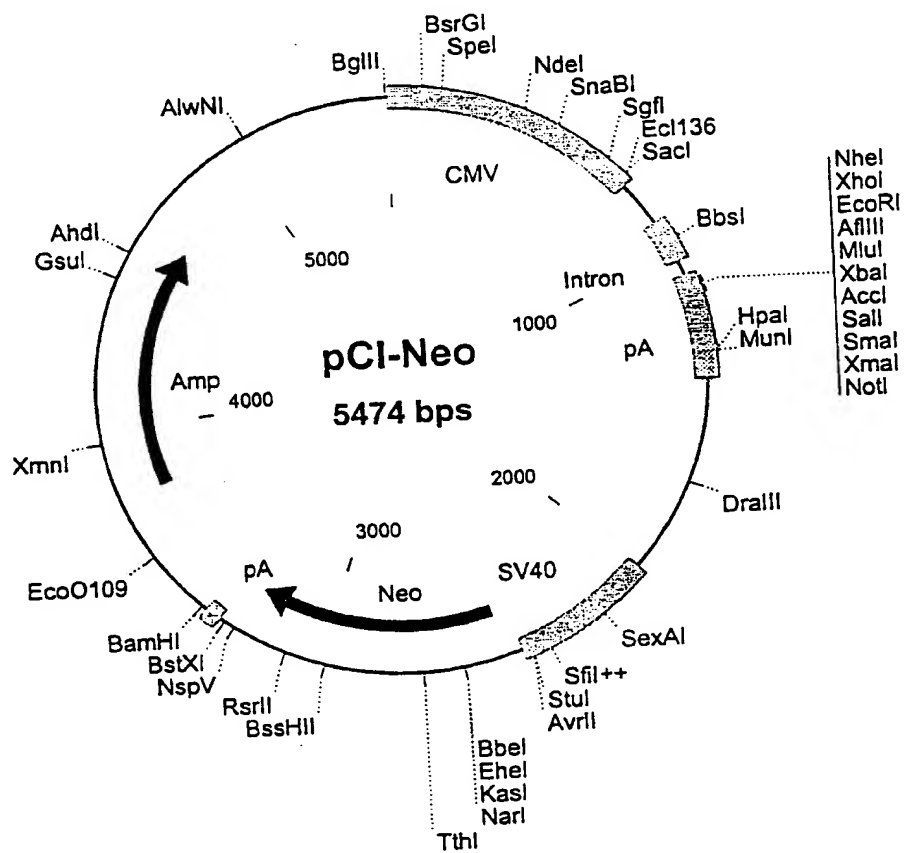
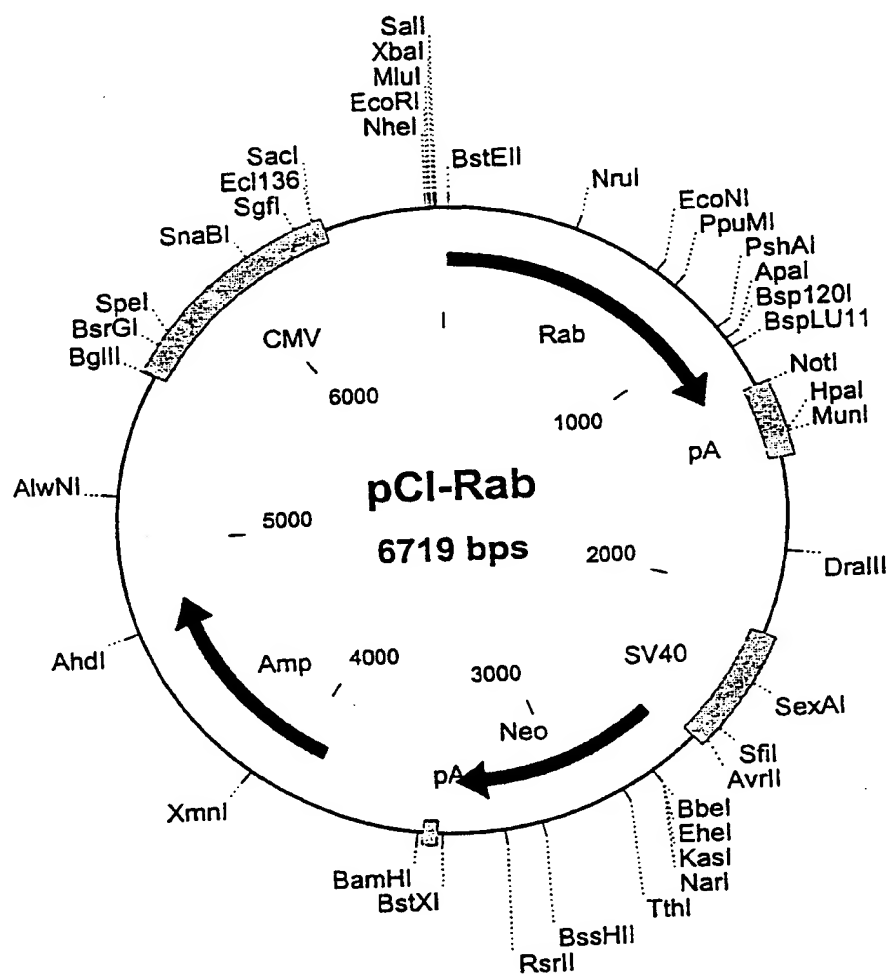




Fig 25



5

Fig 26

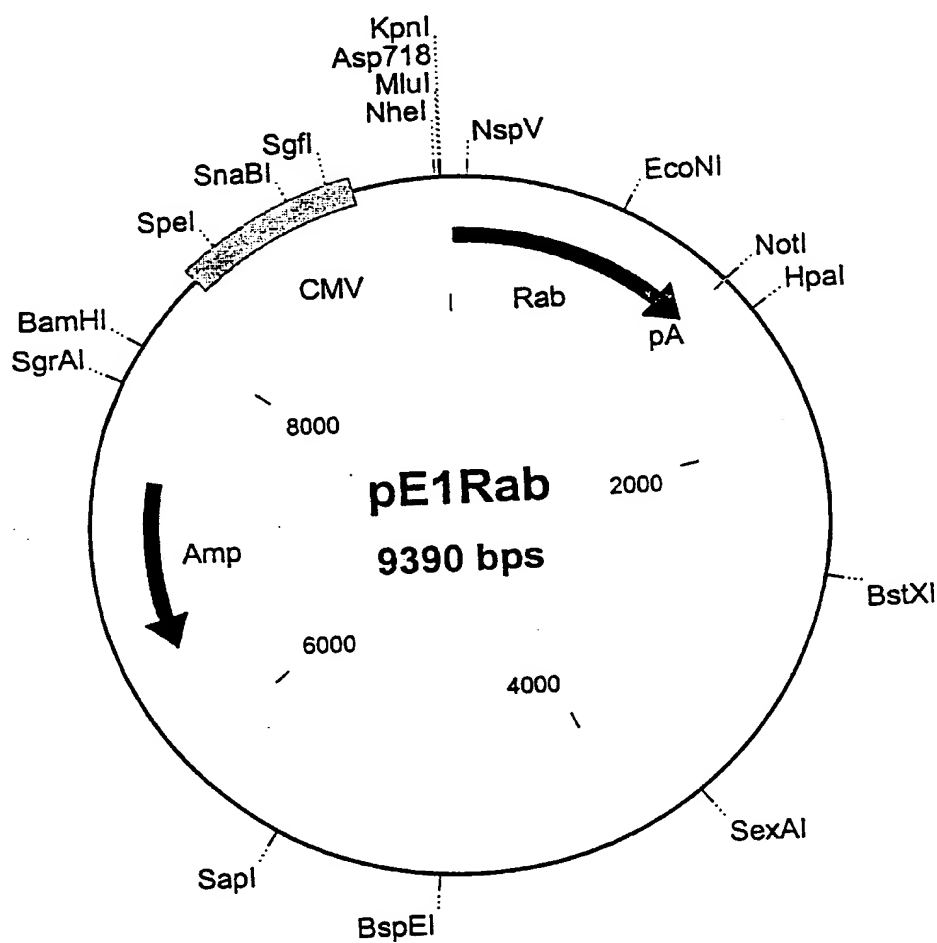
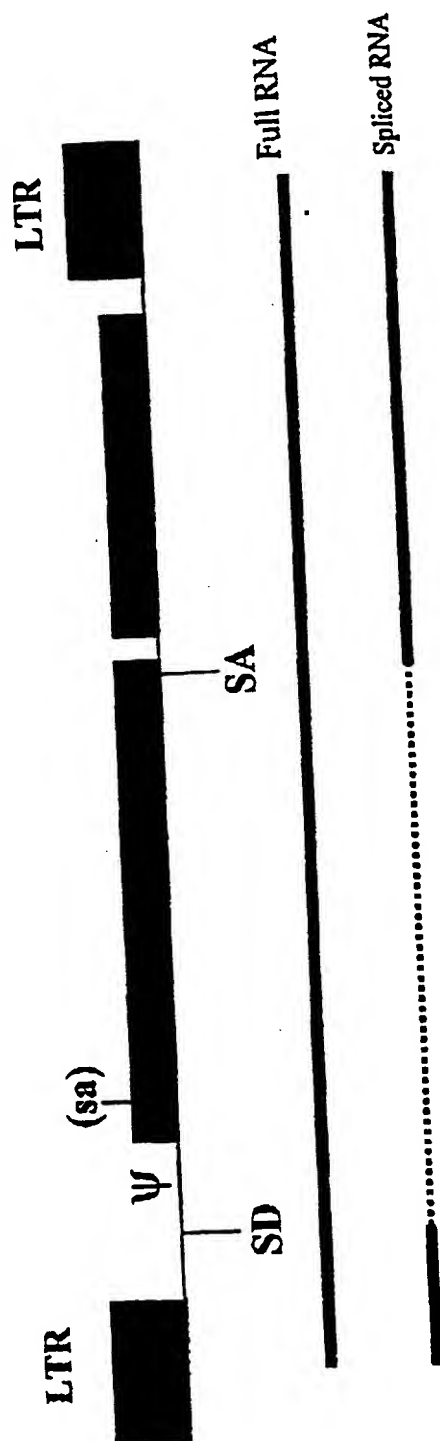


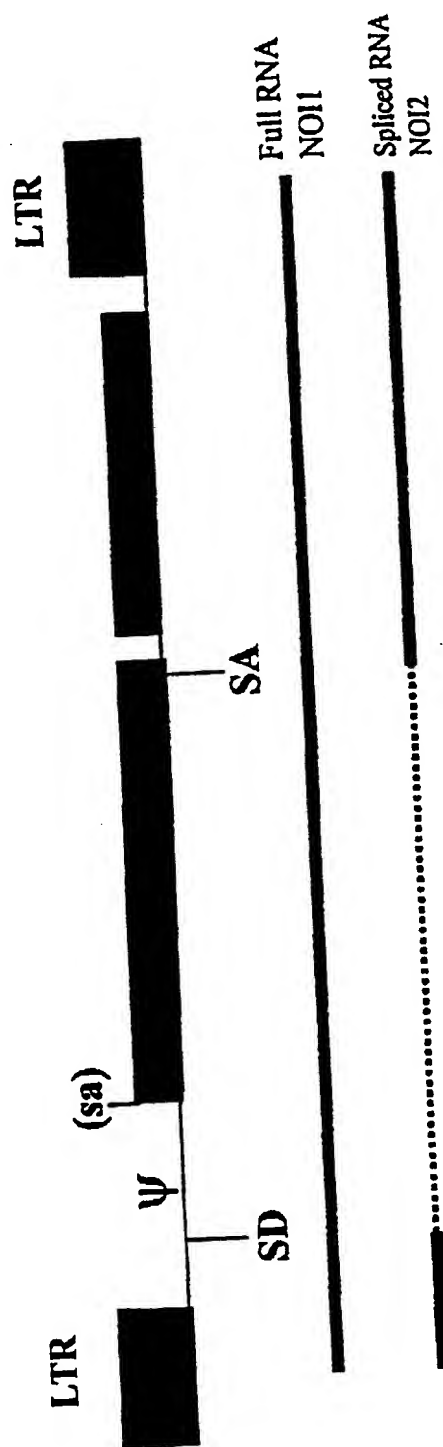
Figure 27a

A)



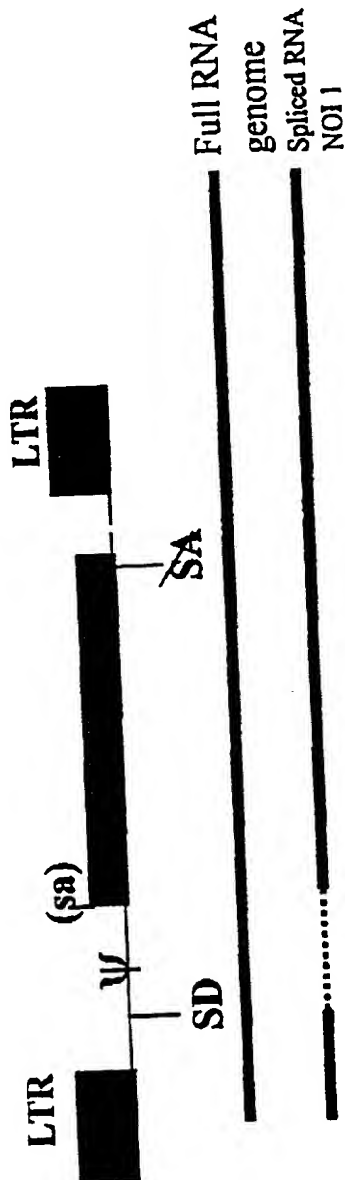
SD = Splice donor  
SA = Splice acceptor  
(sa) = cryptic splice acceptor  
 $\psi$  = packaging site

Figure 27b



SD = Splice donor  
 SA = Splice acceptor  
 (sa) = Cryptic splice acceptor  
 ψ = packaging site

Figure 27b cont:



SD = Splice donor  
 SA = Splice acceptor  
 (sa) = cryptic splice acceptor  
 $\psi$  = packaging site  
~~SA~~ = non functional splice acceptor

e.g. MFG

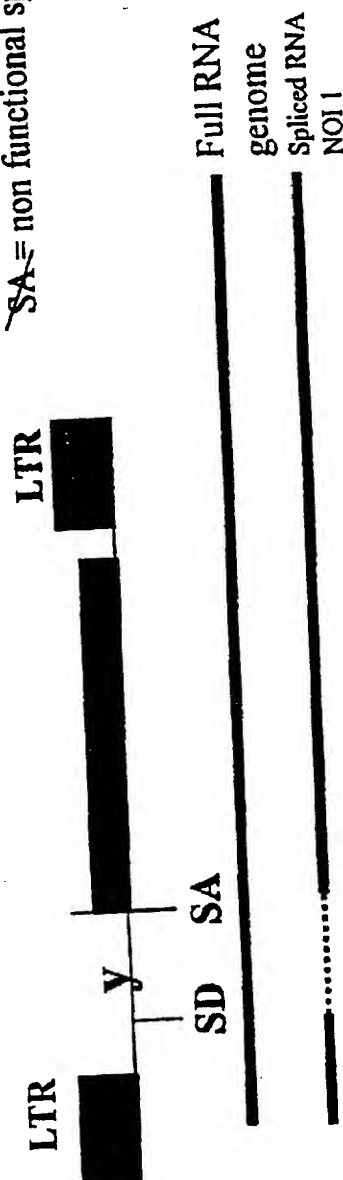
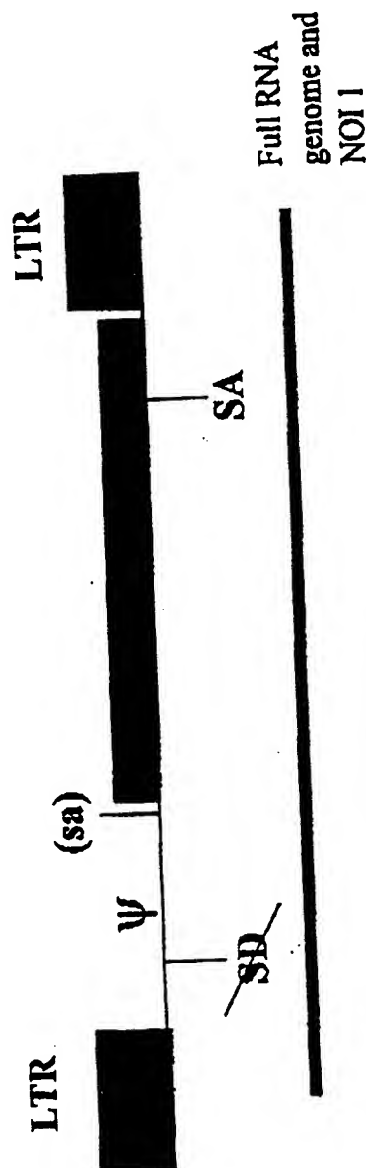


Figure 27b cont



~~SD~~ = Non functional splice donor  
 SA = Splice acceptor  
 (sa) = cryptic splice acceptor  
 ψ = packaging site

SD = Splice donor  
~~SD~~ = non functional splice donor  
 SA = Splice acceptor  
 (sa) = cryptic splice acceptor  
 $\psi$  = packaging site  
 IRES = internal ribosome entry site (optional)

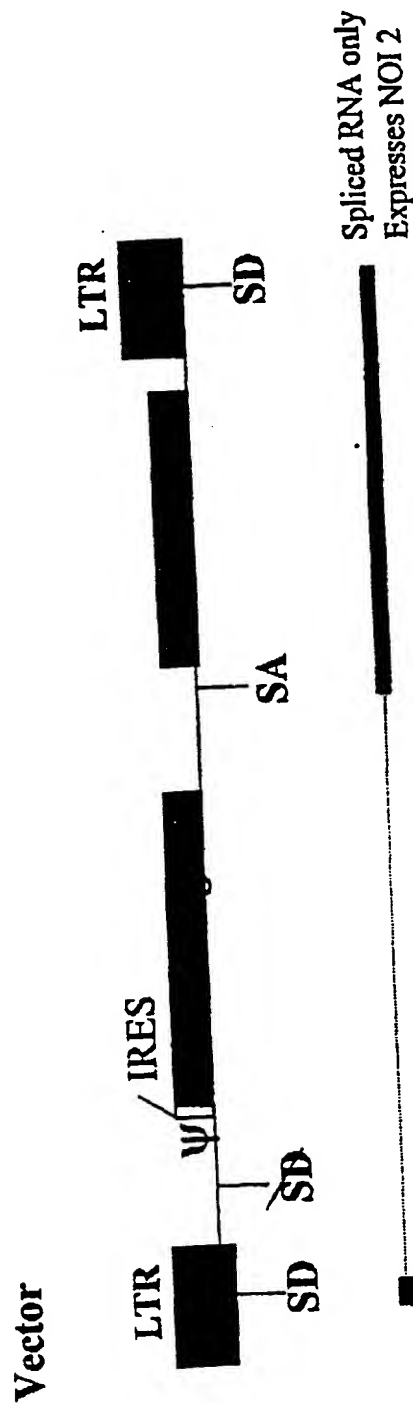
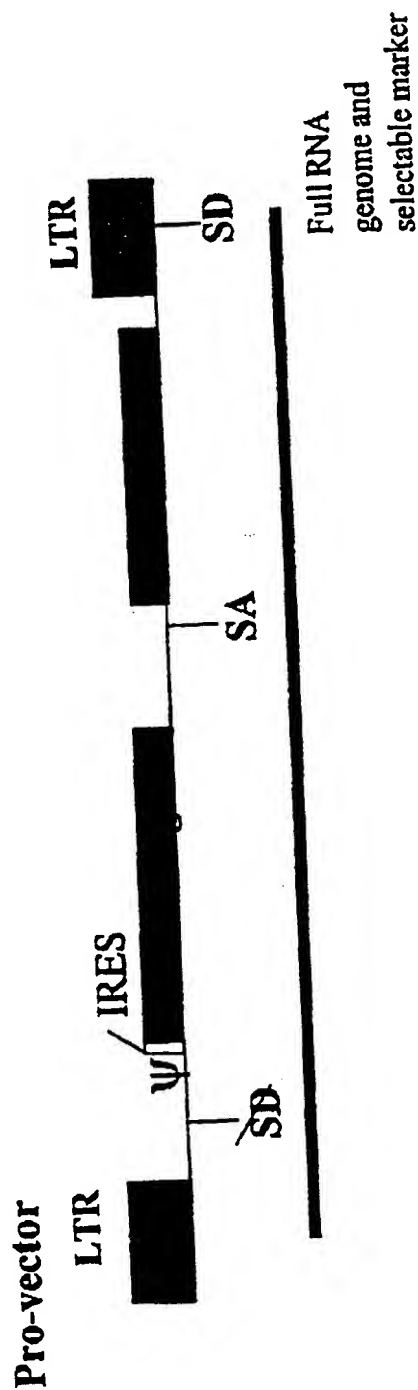
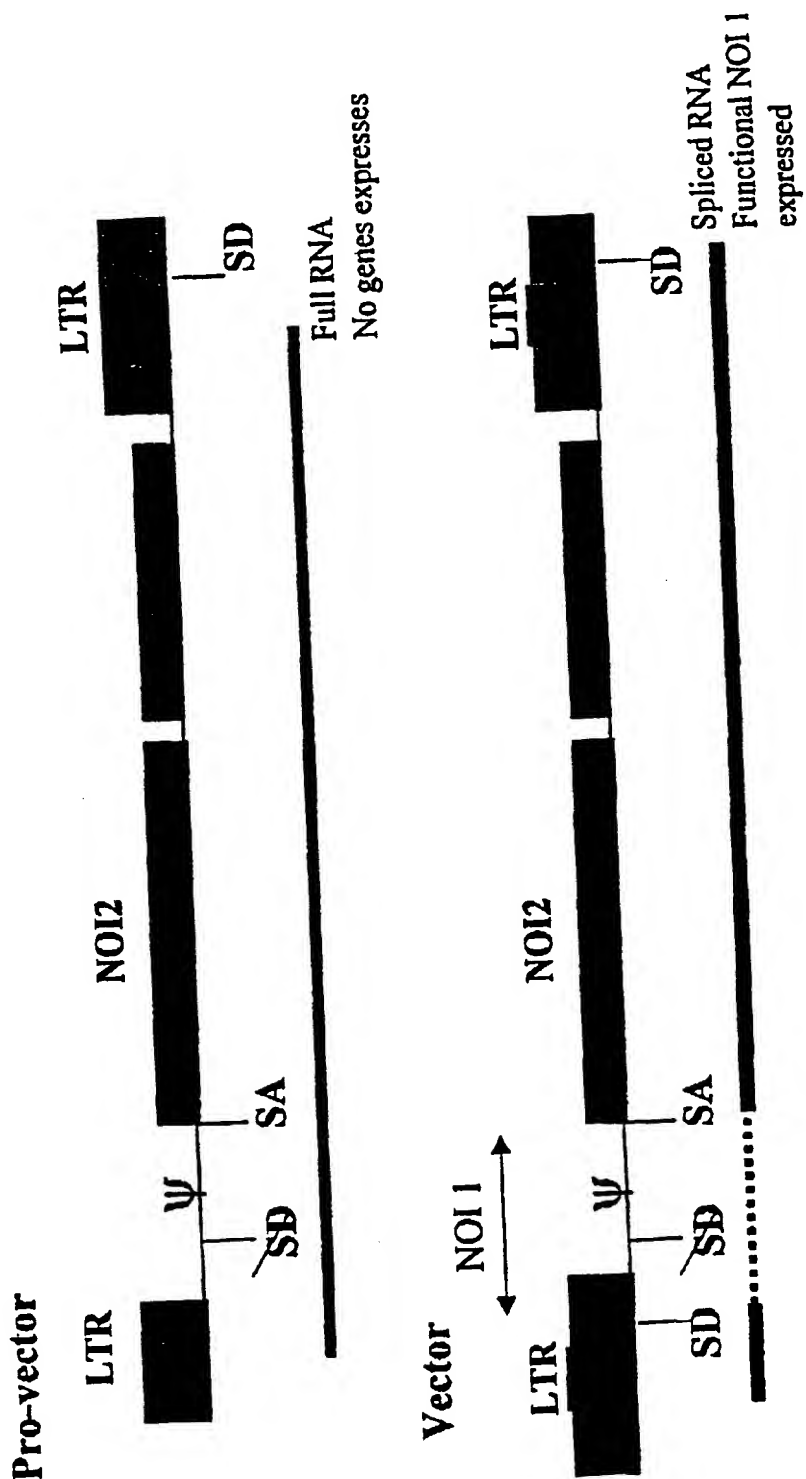


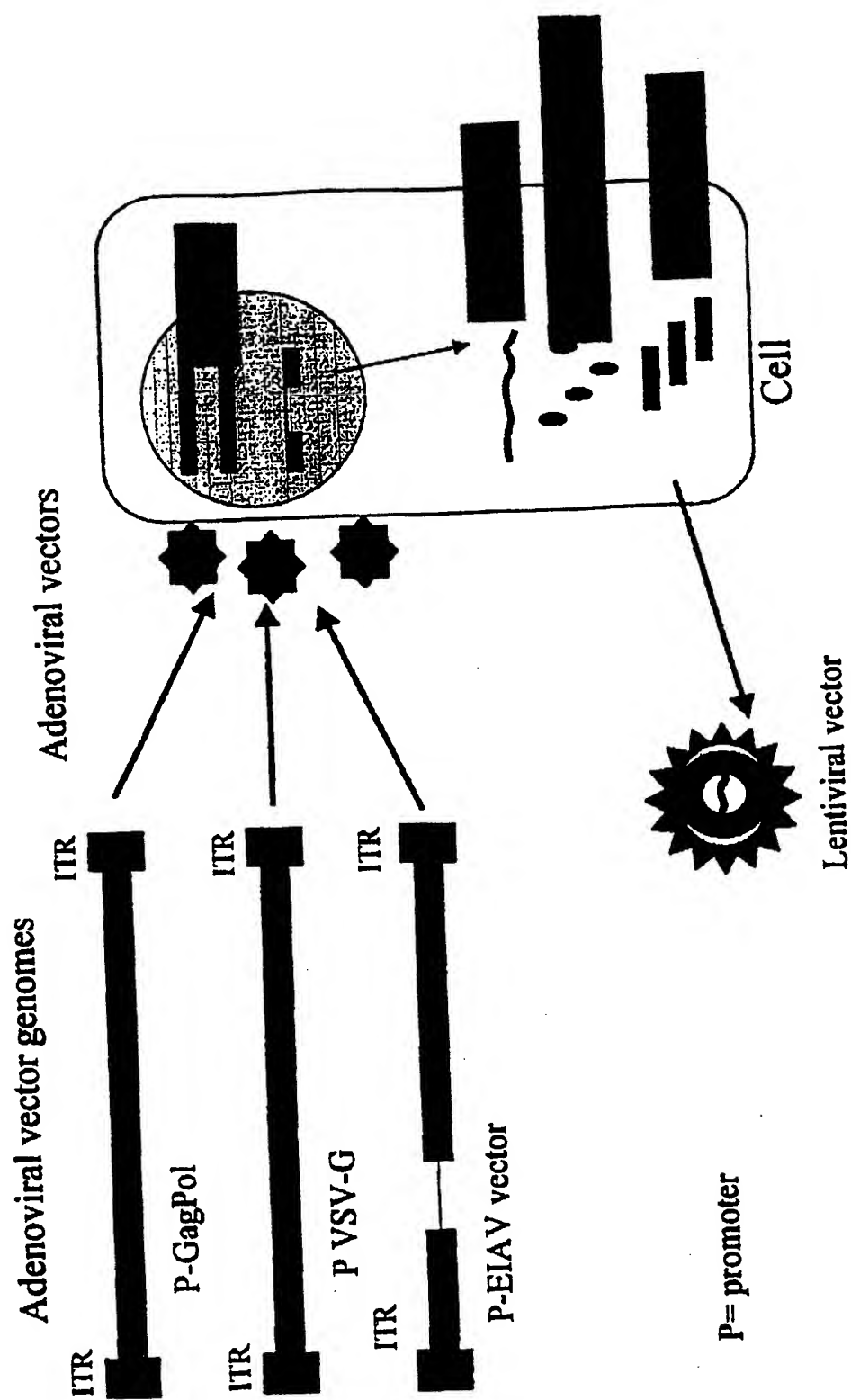
Figure 27c

Figure 27c cont.



NOI3 is optional, if expression is required then an IRES is placed immediately upstream



**Figure 28**

## ANNEX

SEQUENCE LISTINGS

SEQ ID NO. 1

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35 CAAATGGCGATTACCGTTGATGTTGAAGTGGCGAGCGATACACCGCATCCGGCGCGGATTGGCCTGAACCTGCC  
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GCCGCTACAGTCAACAGCAACTGATGGAACACAGCCATCGCCATCTGCTGCACGCGGAAGAGGCATGGCT  
40 GAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGCCGTCAGTATCGGCGGAATTCCAG  
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AGATCCGGCTGTGGAATGTGTGTGATGAGTTAGGGTGTGGAAGTCCCCAGGCTCCCCAGCAGGCAGAGTATGCA  
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GAGGGGTTTTATAAATGATTATAAGAGTAAAAAGAAAGTTGCTGATGCTCTCATAACCTTGTATAACCCAAAG  
45 GACTAGCTCATGTTGCTAGGCAACTAAACCGCAATAACCGCATTTGTGACGCGAGTTCCCCATTGGTGACGCG  
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5 AGTCCCTTCTCTGTCTGGGCTGAAAAGGCCTTTGTAATAAATATAATTCTCTACTCAGTCCCTGTCTCTAGTTT  
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30 TTGCGGCATTTTGCCTTCTGT TTTTGTCTACCCAGAAACGCTGGTGAAAGTAAAGATGCTGAAGATCAGTT  
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CTTCAAGAACTCTGTAGACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGC  
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55 See Figure 16